Investigation of *Staphylococcus aureus* intracellular survival strategies using a photoconvertible reporter system as biosensor for bacterial metabolic state

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II. ABSTRACT

Staphylococcus aureus is an important human pathogen with an extracellular/intracellular dual lifestyle that enables this microorganism to invade, survive and proliferate in host cells, protected from the host immune system and antibiotic treatment, with dramatic consequences regarding treatment failures and recurrent infections. A better understanding of *S. aureus* intracellular lifestyle may facilitate the development of new therapeutic approaches to eliminate *S. aureus* from its intracellular niche. Therefore, the overall aim of this study was to investigate the mechanisms used by *S. aureus* to survive and replicate intracellularly within different host cells. For this purpose, an *S. aureus* strain encoding the reporter mKikumeGR that enables to monitor the metabolic activity and proliferation status of the internalized bacteria was used to simultaneously assess gene expression changes in host cells and pathogens in a dual RNA-seq approach and determine the correlation between these changes and the metabolic state of internalized bacteria. The host transcriptional profiles, which were dominated by the expression of pro-inflammatory genes, did not differ between macrophages harboring metabolically active/ proliferating green-fluorescent *S. aureus* and those harboring metabolically inactive red-fluorescent *S. aureus,* suggesting that the metabolic state of the internalized bacteria was not dictated by heterogeneity within the host cell population. On the pathogen side, metabolically active/proliferating and metabolically inactive *S. aureus* exhibited a common response comprising a core set of genes associated with amino acid biosynthesis, transport systems, virulence factors and regulatory systems, representing a general stress response to the intracellular host milieu. Beside the common response, a transcriptional signature that was specific to the bacterial metabolic state was identified. While metabolic active intracellular *S. aureus* showed higher expression of genes involved in proliferation, the red metabolically inactive bacteria had higher expression of oxidative stress-related genes and ribosome hibernation. The influence of the internalization route on the fate of intracellular *S. aureus* was also investigated in this study. The internalization pathway used by *S. aureus* to access an intracellular compartment permissive for bacterial replication was found to be cell-type specific. While in macrophages, *S. aureus* used an $\alpha 5\beta 1$-integrin-mediated internalization pathway likely via dorsal ruffling and macropinocytosis, the invasion mechanism in epithelial cells seems to be caveolae-mediated and induced by secreted Hla. Furthermore, the polarization state of macrophages influenced the fate of intracellular *S. aureus* since, in contrast to M1 polarized macrophages, only M2 polarized macrophages were permissive for intracellular proliferation.

In summary, this study provides evidence for a cell-type specific internalization pathway of *S. aureus* where clathrin-independent endocytic vesicles might display a permissive compartment for intracellular survival/replication of *S. aureus*.

III. ZUSAMMENFASSUNG

Staphylococcus aureus ist ein wichtiges Humanpathogen mit einem dualen intra-/extra-zellulären Lebenszyklus der diesen Mikroorganismen erlaubt Wirtszellen zu invadieren um dort, geschützt vor dem Immunsystem des Wirts und Antibiotikabehandlungen, zu überleben und sich zu teilen, mit dramatischen Konsequenzen in Bezug auf Behandlungsmisserfolge und wiederkehrenden Infektionen. Ein besseres Verständnis über den intrazellulären Lebenszyklus von *S. aureus* könnte die Entwicklung neuer therapeutischer Ansätze zur Eliminierung des Erregers aus seiner intrazellulären Nische fördern. Daher war es das Ziel dieser Studie, die von *S. aureus* verwendeten Mechanismen zum intrazellulären Überleben in verschiedenen Wirtszellen zu untersuchen. Hierfür wurde ein *S. aureus* Stamm verwendet, der den Reporter mKikumeGR exprimiert, welcher erlaubt die metabolische Aktivität und den Proliferationsstatus der intrazellulären Bakterien zu verfolgen. Dieses Reporter-System wurde genutzt, um Änderungen der Genexpression in den Wirtszellen und dem Pathogen parallel in einem dualen RNA-Seq Ansatz zu bestimmen und diese mit dem metabolischen Status der internalisierten Bakterien zu korrelieren. Die Transkriptionsprofile des Wirts, die hauptsächlich die Expression von pro-inflammatorischen Genen zeigen, unterschieden sich nicht zwischen den Makrophagen, die hauptsächlich metabolisch aktive, proliferierende, grün-fluoreszierenden *S. aureus* und denen die hauptsächlich metabolisch inaktive, rot-fluoreszierende Bakterien enthielten. Daraus lässt sich schließen, dass der metabolische Status der Bakterien nicht zurückzuführen ist auf eine Heterogenität innerhalb der Wirtszellpopulation. Die metabolisch aktiven und inaktiven *S. aureus* exprimierten ein gemeinsames, grundlegendes Set an Genen, die sich der Aminosäuresynthese, der Virulenz, den Transport-, oder Regulationssystemen zuordnen lassen und damit die allgemeine Stressantwort auf das intrazelluläre Milieu darstellen. Außerdem konnten spezielle transkriptionelle Signaturen einzelnen bakteriellen Stoffwechselzuständen zugeordnet werden. Während metabolisch aktive intrazelluläre *S. aureus* wichtige Gene für die Proliferation stärker exprimierten, zeigen metabolisch inaktive *S. aureus* eine stärkere Expression von Genen, die auf oxidativen Stress und den Ruhezustand von Ribosomen hinweisen. Zudem wurde der Einfluss des Aufnahmeweges in die Wirtszellen auf das Überleben der aufgenommenen *S. aureus* im Rahmen dieser Studie untersucht. Es konnte gezeigt werden, dass das Bakterium einen zelltypabhängigen Weg nutzt um ein Kompartiment zu erreichen, in dem es überleben und sich teilen kann. Während eine $\alpha 5\beta 1$-Integrin vermittelte Aufnahme vermutlich über Dorsal Ruffles und Macropinocytose genutzt wird um in Makrophagen zu gelangen, scheint die Invasion von Epithelzellen induziert durch sekretiertes Hla über Caveolae abzulaufen. Außerdem wird das intrazelluläre Überleben von

S. aureus durch die Polarisation von Makrophagen beeinflusst, da im Gegensatz zu M1 Makrophagen nur M2 Makrophagen die intrazelluläre Replikation zuließen.

Zusammengefasst liefert diese Studie Beweise für einen Zelltyp-spezifischen Aufnahmeweg von *S. aureus* bei dem Clathrin-unabhängige endozytische Vesikel Kompartimente darstellen könnten, die das intrazelluläre Überleben/Replikation von *S. aureus* zulassen.

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°C	degree Celsius smaller than
®	registered
±	plus-minus sign
<	smaller than
>	bigger than
3'	three prime end
5'	five prime end
AB/AM	antibiotic-antimycotic
abx	antibiotics
ad	*ad* (Latin 'up to')
ADAM-10	a disintegrin and metalloprotease domain-containing protein
agr	acessory gene regulator
AIP	auto-inducing peptide
AK	antibody
APC	allophycocyanin
ATCC	American Type Culture Collection
B. subtilis	*Bacillus subtilis*
BAM	Binary Alignment Map
BCAA	brached-chain amino acids
BHI	brain-heart-infusion
BMDM	bone marrow derived macrophages
bp	base pair(s)
BSA	serum albumin bovine
C. pneumoniae	Chlamydia pneumoniae
C3a	complement factor 3a
C3b	complement factor 3b
C5a	complement factor 5a
Ccl	C-C motif ligand
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CFU	colony forming unit
CHIPS	chemotaxis inhibitory protein of *S. aureus*
CLSM	convocal laser scanning microscopy

CO_2	carbon dioxide
csv	comma-seperated value
CTR	control
CWA	cell-wall anchored
Cxcl	C-X-C motif ligand
CXCR	C-X-C chemokine receptor
de novo	de novo (Latin 'from the beginning')
DEG(s)	differentially expressed genes
dest.	destilled
DIC	differential interference contrast
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
DR	dorsal ruffle
DSMZ	German Collection of Microorganisms and Cell Cultures
dsRNA	double-stranded ribonucleic acid
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
et al.	*et alii* (Latin 'and others')
FACS	fluorescence-activated cell sorting
Fc	fragment crystallizable
FCS	fetal calf serum
FESEM	Field Emission Scanning Electron Microscopy
FITC	fluorescein isothiocyanate
FnBP	fibronectin-binding protein
g	gram(s)
ɣ	gamma
h	hour(s)
h.i.	heat-inactivated
H_2O_2	hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HRP	horseraddish peroxidase
Hsp(s)	heat shock proteins
HTML	Hypertext Markup Language

ICAM	intercellular adhesion molecule
IFN	interferone
IgG	immunoglobulin G
IL	interleukin
IMΦ	immortalized macrophage(s)
KCL	Potassium chloride
kDa	kilodalton
KEGG	Kyoto encyclopedia of genes and genomes
kg	kilogram
KH_2PO_4	Potassium dihydrogen phosphate
L	liter
LDH	lactate dehydrogenase
LED	light-emitting diode
log	logarithm
LPS	lipopolysacchride
LTA	lipoteichoic acids
LV	low viscosity
M	molar
M-CSF	macrophage colony-stimulating factor
MAMP(s)	microbe associated molecular pattern
Mbp	mega base pairs
MHC	major histocompatibility complex
min	minute(s)
mL	mililiter
mM	milimolar
MOI	multiplicity of infection
mRNA	messanger ribonucleic acid
MRSA	methicillin-resistant *Staphylococcus aureus*
MSCRAMM	microbial surface components recognizing adhesive matrix molecules
MW	molecular weight
MβCD	methyl-β-cyclodextrin
$Na_2HPO_4 \cdot 2H_2O$	sodium phosphate dibasic dihydrate
NaCl	sodium chloride

NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
NaOH	sodium hydroxide
NCTC	National Collection of Type Cultures
NEAT	near iron transporter
ng	nanogram
NGS	next generation sequencing
NLR	(NOD)-like receptor
nm	nanometer
NO	nitric oxide
NOD	nucleotide-binding oligomerization domain
ns	no statistical significance
o/n	overnight
O_2^-	superoxide anion
OD_{600}	optical density at 600nm
ORSA	oxacillin-resistant *Staphylococcus aureus*
p value	probability value
p.I.	post infection
PALM	Photoactivated Localization Microscopy
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PC1	principal component 1
PC2	principal component 2
PCA	principal component analysis
PCR	polymerase chain reaction
PE	phycoerythrin
PFT	pore forming toxin
pH	*potentia Hydrogenii*
PLEKHA7	Pleckstrin Homology Domain Containing A7
PMN	polymorphonuclear neutrophilic leukocytes
PRR	pattern recognition receptor
PSGL	P-selectin glycoprotein ligand
PSM	phenol-soluble modulin
PVL	Panton Valentine toxin
QC	quality control

qRT-PCR	Real-Time Quantitative Reverse Transcription PCR
RBS	ribosome binding site
RIN	RNA integrity number
RLR	RIG-1 like receptor
RNA	ribonucleic acid
rpm	rotations per minute
RPMI	Roswell Park Memorial Institute
rRNA	ribosomal ribonucleic acid
RT	room temperature
RTK	receptor tyrosine kinase
S. aureus	*Staphylococcus aureus*
S. pyogenes	*Streptococcus pyogenes*
SaCP	*S. aureus* containing phagosome
SAK	staphylokinase
SD	standrad deviation
SDS	Sodium Dodecyl Sulfate
sec	seconds
SERAM	secretable expanded repertoire adhesive molecule
SN	supernatant
SSL	superantigen like protein
ssRNA	single-stranded ribonucleic acid
SSS	scalded skin syndrome
STAR	Spliced Transcripts Alignment to a Reference software
STORM	Stochastic Optical Reconstruction Microscopy
TAE	Tris-Acetat-EDTA
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween20
TCA	tricarboxylic acid
TCS	two-component system
TE	Tris-EDTA
TEM	Transmission Electron Microscopy
TGF	transforming growth factor
TLR	toll-like receptor
TM	trademark
TNF	tumor necrosis factor

TPM	transcripts per million
TSS	toxic shock syndrome
U	units
UV	ultraviolet
V. cholerae	*Vibrio cholerae*
V/V	volume/volume
VST	variance-stabilizing tarnsformation
w/o	without
WT	wild type
W/V	weight/volume
x g	g-force
α	alpha
β	beta
δ	delta
µ	micro
µm	micrometer

1 INTRODUCTION

1.1 Staphylococcus aureus

1.1.1 Background

Staphylococci from the phylum Firmicutes are gram-positive, non-spore-forming, nonmotile, spherical bacteria with an average diameter of 1 µm and a facultative anaerobe metabolism[1]. The coagulase positive opportunistic human pathogen *Staphylococcus aureus*, named for its grapelike cluster formation and golden (Latin, '*aureus*') pigmentation, is one of the major causes for community- and hospital-acquired (nosocomial) infections but can also be found on food as well as in association with livestock[2–6]. It was first described by Alexander Ogston in 1882 for its direct link to wound suppuration and sepsis[7,8]. *S. aureus* is extremely resistant against heat[9], UV-light[10], pH alteration[11], and salinity[12]. Although, *S. aureus* can cause a wide range of infections[13], it can also colonize the human skin and mucosa, especially the nasal anterior nares, without any symptoms[14,15]. Thus, around 20 % of individuals are persistently colonized by *S. aureus* while 60% are intermittent carriers[16]. This asymptomatic carriage of *S. aureus* is a major risk for infection[17,18] as patients with *S. aureus* bacteremia are usually infected with their own colonizing endogenous strain[19,20]. Several studies have reported that colonization with *S. aureus* is dependent on different factors including age[21,22], gender[23], underlying diseases[24–26], immunodeficiency[27], and also race[28].

S. aureus is highly adapted to the human host and exhibit a high pathogenic potential that is also reflected by its genome. Most *S. aureus* strains have a G+C poor genome of around 2.8Mbp, of which around 80% is highly conserved between different strains[29]. Many variable or accessory genes such as antibiotic resistance genes and other genes expressing virulence factors are generally found in genomic islands that have been mostly acquired by horizontal gene transfer[30]. *S. aureus* exhibits a remarkable capacity to adapt to the different microenvironments encountered during infection that is facilitated by its high genomic plasticity through lateral gene transfer from other species[31].

1.1.2 Infections caused by *S. aureus*

S. aureus can cause a variety of infections in humans that can range from mild to severe forms[13] and is a leading cause of bacterial infections worldwide[32,33]. It is the predominant bacterial pathogen of nosocomial respiratory tract and surgical site infections[34,35] and,

especially infections with methicillin- and oxacillin-resistant *S. aureus* (MRSA/ORSA) strains with resistance against a broad spectrum of antibiotics, are highly problematic in intensive care units[1]. Moreover, bloodstream infections, pneumonia or cardiovascular infections are often caused by *S. aureus*[34]. Local infections at the skin such as acne, impetigo or boils are some examples of infections caused by coagulase-positive strains, mostly common among children and are relatively harmless[4,36]. After a break in the protective barrier of the skin or other epithelial surfaces, either by surgical operation or wounds, *S. aureus* can enter the bloodstream and access deeper tissues causing invasive infections such as meningitis, sepsis, bacteremia, osteomyelitis and pneumonia[36–38]. Besides invasive infections, toxin-mediated diseases like scalded skin syndrome (SSS) or toxic shock syndrome (TSS) are also caused by *S. aureus*[13]. Main risk factors for such invasive infections are catheters, implanted devices and prostheses as the bacteria can attach to the surfaces of these devices and form biofilms[39]. A hematogenous distribution of *S. aureus* can also lead to the establishment of metastatic infections[38] followed by long-lasting persistent or recurrent infections that are often therapy-refractory[40]. Another major problem often associated with *S. aureus* infections is the overshooting inflammatory reaction of the host immune system, which is highly destructive and can lead to sepsis.

1.1.3 *S. aureus* Pathogenicity

A main reason for the remarkable success of *S. aureus* as a pathogen is the expression of a wide array of virulence determinants promoting host colonization, survival in the bloodstream or deeper tissues and evasion of the host innate immune response[36,41–43]. These factors can roughly be categorized in surface proteins, including cell-wall anchored proteins and non-covalently adhesive proteins, and secreted factors, including secreted enzymes, toxins, and invasins. The expression of these factors is tightly regulated and controlled by a whole network of regulatory systems that enable *S. aureus* to adapt to different environments at the site of infection and to overcome the environmental stresses encountered during infection[44,45].

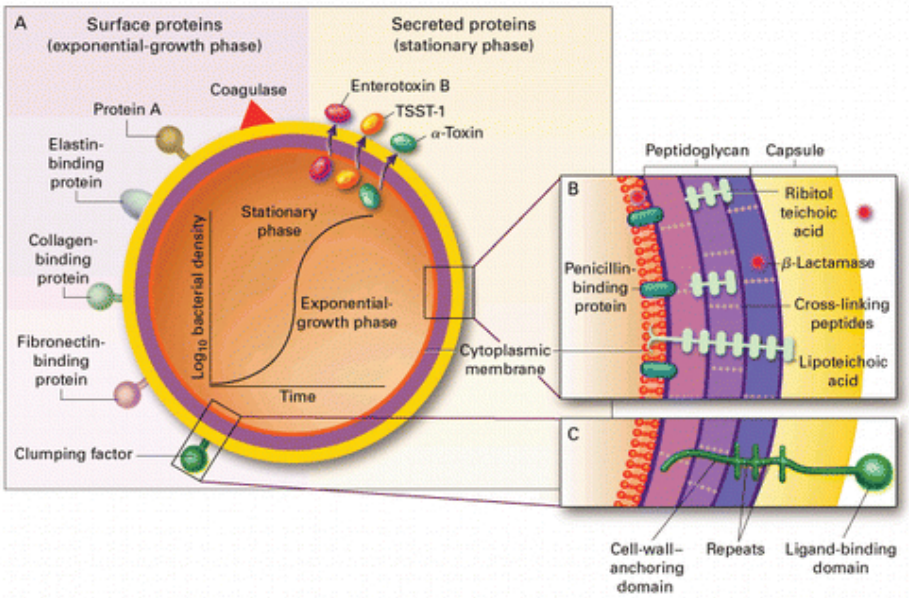

Figure 1 |Selection of *S. aureus* virulence determinants.
Surface and secreted *S. aureus* proteins (A) as well as cross-sections of the cell envelope (B and C) that play a role as virulence factors. Adapted without modifications from Lowy, 1998[13].

Cell-wall anchored proteins

S. aureus can express up to 24 different cell-wall anchored (CWA) proteins involved in adhesion to host cells and tissues, host invasion, and innate immune evasion and are crucial for bacterial survival and spreading during infection[46–48]. The expression of specific CWA proteins varies among the different *S. aureus* strains and is dependent on growth conditions and growth phase[47,49]. It has been shown that one specific CWA protein can have multiple functions and many of them show functional redundancy[46]. Translated precursor CWA proteins have an N-terminal secretory signal sequence that directs them to the secretory (sec) apparatus where they are cleaved and exported[46,50]. The precursor proteins also contain a C-terminal sorting signal that facilitates their covalent linkage to cell wall peptidoglycan by the transpeptidase activity of sortase A and B[51–53]. Based on structural motifs, it has been proposed to classify CWA proteins into four groups, with the microbial surface component recognizing adhesive matrix molecule (MSCRAMM) family being the largest one[46]. The current definition of MSCRAMMs includes proteins with structural and functional similarities and a common mechanism for ligand binding mediated by at least two adjacent IgG folded domains at the N-terminus[46]. This description includes both clumping factors A and B (ClfA, ClfB), the serine-aspartate repeat proteins (SdrC, SdrD, SdrE), the fibronectin-binding proteins A and B (FnBPA, FnBPB), and the collagen-boning protein (Cna)[46]. Especially the clumping factors A and B that promote adhesion to immobilized fibrinogen (ClfA) and epithelial cells (ClfB) have been well studied[54–56]. Another function of ClfA is the degradation of C3b by binding the complement

factor I, resulting in impaired opsonization and thus decreased phagocytosis[57,58]. The role of the other aforementioned proteins is less understood but has been shown to play a role in the initial adhesion and invasion of different host cell types[59–63]. The importance of MSCRAMMs and their role as virulence factors has been validated in animal models of endocarditis (e.g. ClfA[64], ClfB[65], FnBPA[66]), septic arthritis (e.g. Cna[67], ClfA[68,69]), and kidney abscesses (e.g. SdrD, SdrE. ClfA, ClfB)[70].

The other families of the CWA proteins are the NEAT motif family (IsdA, IsdB, IddH), the three-helical bundle protein A (Spa), and the G5-E repeat family (Sas family)[46]. Near iron transporter (NEAT) proteins are mainly involved in iron acquisition to survive within the host under iron limiting conditions during infection[48,71,72]. However, recent studies have reported an additional role in invasion of non-phagocytic cells[73], resistance to bactericidal lipids and antimicrobial peptides[74,75] and degradation of the C3b opsonin[76]. Protein A mediates the inhibition of opsonophagocytosis[77] and has pro-inflammatory effects[78]. The staphylococcal surface protein SasG promotes adhesion to epithelial cells and plays a role in biofilm formation[79].

Secreted adhesive proteins

In addition to the cell-wall anchored proteins, also secreted proteins that attach non-covalently to the bacterial surface after or during secretion can act as adhesins. These secretable expanded repertoire adhesive molecules (SERAMs) bind with broad specificity to various host effector proteins[80,81], including the matrix proteins fibrinogen, fibronectin, vitronectin or collagens, as well as plasma proteins like thrombospondin[82–84], and thereby interfering with defense mechanisms of the host.

The extracellular adherence protein (Eap)[85], also called MHC class II-analogous protein (Map)[86], which is highly prevalent in clinical isolates of *S. aureus*[83], can bind these matrix and plasma proteins and has an additional immune-modulatory function by interacting with the ICAM-1 receptor that is also essential for neutrophil attachment to the endothelium[87]. This interaction leads to reduced neutrophil recruitment and an impaired wound-healing process[88] and has been validated in animal models of peritonitis and renal abscess formation[87,89]. Moreover, Eap has been shown to promote *S. aureus* internalization in fibroblasts and epithelial cells[90].

Other secreted adhesins are the less well-understood extracellular matrix-binding protein homolog (Ebh) and the extracellular matrix binding protein (Emp), both showing high binding affinity to the extracellular matrix components vitronectin, fibrinogen, and fibronectin[82,85]. Although their functional role *in vivo* is still unclear, Emp, with a notable very

high affinity for vitronectin, has been suggested to play a role in the recruitment of factors involved in various processes, like complement activation, tissue remodeling, or homeostasis[84]. Furthermore, Ebh has been speculated to have an important function in hyperosmotic stress tolerance[91].

Toxins

Over 40 secreted toxins (exotoxins) are known to be produced by *S. aureus*, with high structural similarities between them but very unique properties ranging from host cell damage, to disband cell junctions or immunomodulatory activities[92,93]. While most of these toxins are encoded on mobile genetic elements, the hemolysins, the phenol soluble modulins, and the superantigen selX are located on the core genome of *S. aureus*[92]. Based on their function, staphylococcal toxins can be roughly divided into the following three groups: (i) cytotoxins, lysing target cells and resulting in inflammation; (ii) superantigens, mediating T and B cell proliferation and massive cytokine production and (iii) secreted cytotoxic enzymes, damaging the mammalian host cells[92,93]. The group of cytotoxins can be classified into two classes of pore-forming toxins (PFTs), the α-PFTs (e.g. delta-hemolysin) and β-PFTs (e.g. leukocidins)[94,95].

S. aureus expresses a notable collection of PFTs that can directly damage the membrane of host cells and thus causing cell death[96]. Additionally, they can also modulate cellular responses such as induction of pro-inflammatory responses without causing cell death[97]. The most prominent pore-forming toxin and most studied virulence factor of *S. aureus* is alpha-hemolysin (α-toxin; Hla), which gene is present on the genome of 95% of *S. aureus* strains[92]. Secreted as water-soluble monomers, it forms heptameric β-barrel transmembrane pores in the target cell membrane resulting in species-specific cell lysis including epithelial cells, fibroblasts, macrophages, monocytes and lymphocytes[98–104]. Recently, ADAM-10 was identified as a receptor that mediates the initial binding of Hla to the cell membrane[104], but also PLEKHA7 has been shown to be involved in Hla cytotoxicity[105]. Another interaction partner that has been proposed is caveolin-1, present in most mammalian cells. A direct binding site of Hla to the scaffolding domain of caveolin-1 has been also reported and inhibition of Hla-induced hemolysis by caveolin-1 was observed[106,107]. Besides its lytic effect, Hla has several modulatory functions at sublytic concentrations, including induction of nitric oxide release by endothelial and epithelial cells, pyroptosis of monocytes, production of pro-inflammatory cytokines and extracellular Ca^{2+} influx[100,108–112]. Inactivation of Hla has been shown to limit *S. aureus* virulence and its importance has been demonstrated in murine pneumonia, skin infection and abscess formation models[113–115]. Relatives to Hla are the bicomponent pore-forming toxins that share structural homology and a similar pore-forming mechanism but require two subunits

and forms hetero-octameric pores[93,97]. Bicomponent toxins associated with human infections, including ɣ-hemolysin (HlgA/HlgB; HlgC/HlgB), the Panton-Valentine leukocidin (LukF-PV/LukS-PV) and two leukocidins LukE/LukD and LukG/LukH[116–118], have been described to be cytotoxic towards neutrophils[119,120], macrophages and monocytes[97,121] while also having sublytic effects, like extracellular Ca^{2+} influx on host cells[122–124] or pro-inflammatory cytokine production[122,125–129]. Another two bicomponent PFTs, LukMF and LukPQ, have been described in *S. aureus* strains that cause infections in animals[130–132]. The role of bicomponent PFTs in virulence of *S. aureus* has been studied in animal models, demonstrating a contribution to pathogenesis during skin[133] and soft tissue infections[134], pneumonia[135], abscess formation[120], bacteremia[120,121] and septic arthritis[136]. A third group of pore-forming toxins includes the amphipathic peptide family called phenol soluble modulins (PSMs) that are classified based on their length as α-PSMs (PSMα1-4, Hld) which are 20-25 amino acids long and β-PSMs (PSMβ1, PSMβ2) which are 44 amino acids long[137–139]. PSMs have several roles in *S. aureus* pathogenesis, including biofilm formation, immune modulation as chemo-attractants for neutrophils, and cell lysis. PSMα show the highest potency in lysing eukaryotic cells[137,140–143]. The role of PSMs during *in vivo* infection remains unclear, as serum lipoproteins can inhibit their cytotoxicity[144]. Recently, PSMs have gained great interest since it was shown that phagocytized intracellular *S. aureus* produced PSMs to lyse osteoblasts and neutrophils[142,143], and a role for intracellular phagosome escape has been proposed[145].

Regulatory systems

The expression of virulence genes in *S. aureus* is tightly controlled by several regulatory systems. Gene regulation is an essential function for pathogens to rapidly change their gene transcription in response to changes in environmental conditions. *S. aureus* controls its expression of virulence determinants through a complex network of regulatory proteins, regulatory RNAs, and two-component systems (TCS)[45]. Dependent on the growth phase, a shift in the expression of *S. aureus* virulence factors from adhesins during the early phase to exoproteins during the late exponential phase has been observed[45,146,147]. So far, 16 TCS have been identified on the genome of *S. aureus*[148], with agrAC, saeRS, srrAB, arlSR and lytSR systems being associated with the regulation of virulence factors[44]. The most prominent and best-studied TCS is the accessory gene regulator (agr), a quorum-sensing system, acting as a master virulence regulator[44,149]. Agr has a dual-mode of action at the post-exponential phase since it represses several cell wall associated proteins (e.g. FnBPA) and activates various exoproteins (e.g. Hla)[44]. The agr system consists of five genes (*agrB*, *agrD*, *agrA*, *agrC* and *hld*) on two divergent transcripts, RNAII and RNAIII, under the control of two inverse promoters

(P2 and P3)[150,151]. The RNAII operon under the P2 promoter comprises four genes *agrBDCA*, the encoded proteins AgrC and AgrA represent the sensor and the response regulator, respectively[152], and AgrB and AgrD representing an autocatalytic sensory transduction system[150]. Briefly, AgrD, the precursor of the autoinducing peptide (AIP) gets processed by the membrane-bound AgrB, followed by the transport across the membrane into the extracellular environment. Outside the cell, AIP is sensed and bound by AgrC, resulting in signal transduction to the response regulator AgrA, which can bind to promoters P2 and P3 to drive RNAII and RNAIII expression, respectively[153–160]. Another regulatory system that has gained considerable attention since it is involved in response to external stresses is the sae (*S. aureus* expoprotein expression) system comprising a sensor histidine kinase SaeS and a response regulator SaeR[161]. Positively regulated by agr, it induces the expression of several important exoproteins, including coagulase, Hla, LukGH, or β- and ɣ-hemolysins[162–164]. This TCS is regulated through host-specific signals and environmental cues, including low pH, high salt concentrations, copper, and zinc[161].

Coupled with the two-component systems of *S. aureus*, cytoplasmic regulatory proteins are essential for the bacteria to survive within the host environment. The staphylococcal accessory regulator SarA and its homologs, summarized as the SarA protein family, together with the alternative sigma factors (SigB and SigH), are the most important representatives. In general, sigma factors can initiate gene transcription by binding to RNA polymerase forming an RNA polymerase holoenzyme that can recognize specific promoter motifs[44]. While the primary sigma factors are required for expression of housekeeping genes[165], the alternative sigma factors regulate the gene expression upon changed environmental conditions, allowing *S. aureus* to adapt to various environments. SigB responds to different stresses, including starvation, heat shock or osmotic shock, and regulates stress-response proteins[44]. Around 200 genes have been identified to be directly or indirectly under the control of SigB including those associated with functions in virulence, cell internalization, antibiotic resistance, membrane transport, persistence, and biofilm formation[166]. The alternative sigma factor SigH is less well understood. It has been shown that overexpression of SigH is leading to natural competence, which is supported by the fact that also in other bacterial species some competence associated genes are regulated by this factor[167–169]. As SigH also stabilizes the lysogenic state of *S. aureus* prophages, that often carry virulence determinants genes, its role in *S. aureus* virulence may be underrated[161,170].

1.2 Host Immune Defense Mechanisms

The immune response is broadly defined as a complex biological network that enables an organism to recognize foreign substances, toxins or microorganisms and defend itself against them[171]. The combination of the innate and the adaptive immune system provides a system that recognizes and eliminates invading pathogens with maximal efficacy, minimal damage to itself, and additionally providing immunological memory to enable a rapid response to re-infection[172]. Besides the immune system, external defenses also contribute to protection against potentially harmful insults.

1.2.1 External immune defenses

External defenses are the first line of defense against pathogenic microorganisms that the body is continuously exposed to[173,174]. These passive mechanisms include anatomical, physical barriers like the skin and the inner lining mucous membranes of the respiratory tract, the intestine, or the urogenital tract, as well as chemical factors like saliva, tears or bronchial fluids. In order to establish infection, a pathogen needs to overcome these epithelial barriers, either by active binding to epithelial surface molecules or by passive invasion through breached barriers like wounds[173,174]. Pathogens that are able to attach to the epithelium can either infect the host cells or damage the integrity of the epithelial layer to enter deeper tissues. In addition to the physical epithelial barriers and associated antimicrobial compounds, most epithelial surfaces are associated with a normal flora of non-pathogenic bacteria and fungi. This normal flora competes with encountering pathogens for nutrients and attachment sites, and can produce antimicrobial peptides that help to prevent pathogen colonization[174].

Moreover, epithelial cells have been shown to play an important role in initiating the immune response[175–179]. Besides being a physical barrier with secretion of chemical factors like epithelium-derived antimicrobial peptides[180], recent findings suggest that the epithelium act as initial sensor for pathogens, orchestrates the ensuing immune response, and maintains cell integrity at the same time[181]. Although professional phagocytes like macrophages are the main immune cells involved in pathogen uptake and killing, also epithelial cells are able to phagocytize as facultative or non-professional phagocytes, a process that is also important to remove dying cells from the epithelium and maintain membrane integrity[181,182]. Various pathogens use the epithelium as a portal for invasion by triggering its entry through a process called pathogen-induced phagocytosis[183]. Although the general phagosome maturation following pathogen engulfment is relatively similar between professional and non-professional phagocytic cells, the phagolysosome formation is much faster and the number of

lysosomes higher in professional phagocytes resulting in more efficient killing[184,185]. As a result, some intracellular pathogens are able to survive within epithelial cells but not in other cell types like macrophages through escape or evasion of the phagocytic degradation pathway.

1.2.2 The innate immune system

After a pathogen overcomes the external defenses, it is usually immediately recognized by the innate immune system, which is the first line of immune defenses against an invading pathogen and is non-specific[186]. One key aspect of the innate as well as the adaptive immune system is to distinguish self from non-self antigens in order to avoid the elicitation of autoimmune destructive mechanisms[187]. The innate immune system recognizes evolutionary conserved motifs in pathogens called pathogen-associated molecular patterns (PAMPs) leading to the stimulation of inflammatory response and pathogen phagocytosis by immune cells such as macrophages and neutrophils[188]. PAMPs such as peptidoglycan cell wall, bacterial flagella, lipopolysaccharides (LPS) or teichoic acid, are essential for pathogen viability, distinguishable from "self" molecules and highly conserved among a class of pathogens and are recognized by germline-encoded host cell receptors called pattern recognition receptors (PRRs)[173,189–192]. PRRs are expressed by the vascular endothelium and innate immune cells in soluble form as complement system as well as in membrane-bound form as host cell surface or intracellular vesicle receptors[173]. The Toll-like receptors (TLRs) are the most prominent counted among these membrane-bound PRRs, which play an important role in initiating the inflammatory response after pathogen recognition and for the induction of the adaptive immune response. TLRs can be found on the surface of professional phagocytes such as macrophages and neutrophils, as well as lung and gut epithelial cells and within endosomes [186,193,194]. 10 members of the TLR family can be found in human (TLR1-TLR10) and 12 in mouse (TLR1-TLR9, TLR11-TLR13), with TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 localized on the cell surface and TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13 localized in the endosome[195,196]. Besides the TLRs family, recognition of cytosolic pathogens can be accomplished by other PRRs families, including retinoid acid-inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs)[197,198]. The primary PRR involved in *S. aureus* recognition is TLR2 on the plasma membrane of neutrophils, monocytes/macrophages, dendritic cells, mast cells or astrocytes[199], recognizing lipoteichoic acid (LTA) and lipoproteins[200,201]. However, it could be shown that after stimulation of TLR2 with bacterial ligands, the receptor is internalized into endolysosomal compartments and can induce a type I interferon response beside the classical pro-inflammatory response induced from the cell surface[202,203]. The broad spectrum of ligands

recognized by TLR2 resulting from the cooperation with TLR1 and TLR6, forming heterodimers[204]. In this regard, TLR6 has been shown to cooperate with TLR2 in *S. aureus* recognition[204]. However, it has been reported that macrophages from TLR2-deficient mice could still produce inflammatory cytokines in response to *S. aureus* stimulation[205], indicating that other receptors are also involved in the recognition of this pathogen. One of these additional receptors is the receptor for bacterial DNA TLR9, which has been shown to mediate the induction of type I IFN pathway by *S. aureus* in dendritic cells[206]. The cytosolic receptor NOD2 has also been reported to be involved in recognition of intracellular *S. aureus*, most probably by recognizing peptidoglycan of the bacteria cell wall[199].

Cell-mediated innate immune response

Recognition of a pathogen by a phagocytic cell is generally followed by its intracellular uptake. Macrophages and neutrophils as professional phagocytes are among the first cells encountering invading pathogens like *S. aureus*. Both cell types have a key role in innate immunity as they are able to recognize, ingest, and kill pathogens[173,174]. Macrophages are resident in practically all tissues in the body including alveolar macrophages in the lung, Kupffer cells in the liver, microglia in the brain and Langerhans cells in the skin. The tissue-resident macrophages are largely established prenatally and maintaining by self-renewal[207]. However, it has been shown that macrophages have not only an embryonic origin but they can also develop from hematopoietic stem cells in the bone marrow to become monocytes that enter the bloodstream[208,209]. Thus, the resident macrophage population can be replenished by infiltrating monocytes from the circulation. Within the tissue, these infiltrating cells can differentiate into tissue-resident macrophages similar to those of fetal origin[207]. A crucial factor for macrophage differentiation is the monocyte colony stimulating factor (M-CSF)[173,209,210]. Macrophages are quite plastic cells that can change their phenotype and physiology in response to environmental signals[211], with classically activated M1 macrophages representing the one extreme and alternatively activated M2 macrophages the other[209,212–214]. Between these two extremes, with enhanced microbicidal or tumoricidal activity including the secretion of pro-inflammatory factors for M1 macrophages[215,216] and M2 macrophages promoting wound healing and anti-inflammatory signals to balance the immune response, a whole spectrum of macrophages with specific functions and characteristics differentiate as a result of the surrounding environment[212]. Although macrophages can easily phagocytose *S. aureus*, this bacterium have evolved numerous strategies to evade the killing mechanisms of macrophages. These bacterial evasion strategies will be described in the next section.

In contrast to long-lived macrophages, neutrophils, also called polymorphonuclear neutrophilic leukocytes (PMNs), are short-lived cells circulating in the bloodstream and do not enter normal, healthy tissue under homeostatic conditions[173,217,218]. Circulating neutrophils are rapidly recruited to the site of tissue damage or infection in response to chemoattractant signals such as IL-8 produced by local cells such as macrophages, neutrophils or epithelial cells in response to infection[219]. Interestingly, N-formyl peptides or lipoteichoic acid produced by *S. aureus* have been reported to directly induce recruitment of neutrophils[220,221]. Neutrophils are critical for the control of *S. aureus* during infection as demonstrated by the highly susceptibility to severe *S. aureus* infection of individuals with neutrophil defects[222]. Because neutrophils play an important role in the control of *S. aureus*[223], this pathogen produces numerous virulence factors that interfere with the recruitment of neutrophils or limit their functional activity in order to persist within the host[224,225].

Macrophages and PMNs play a primary role removing pathogens through phagocytosis, which is initiated by binding of the pathogen to the cell surface leading to a signaling cascade that culminates with the engulfment of the pathogen in a membrane-enclosed phagosome. After the fusion of a phagosome with one or more lysosomes containing lysozymes and acid hydrolases to a phagolysosome, bacterial cell walls as well as proteins can be degraded. In response to phagocytosis, the production of other toxic substances e.g. hydrogen peroxide (H_2O_2), the superoxide anion (O_2^-) or nitric oxide (NO), called the respiratory burst, is induces and support the phagocytes to kill the pathogen. Phagocytosis efficiency is enhanced when bacteria are opsonized by host opsonic molecules such as complement and antibodies and it is mediated by the binding of opsonized bacteria to complement or Fc receptors, respectively[226,227].

While macrophages usually survive this killing boost, neutrophils mostly die[174,186,188,228]. Another function of macrophages is their immunomodulatory role through the release of cytokines leading other immune cells to the site of infection and activate them. Cytokines and other soluble mediators that are part of innate immunity are classified as the humoral innate immune response.

Humoral innate immune response

Together with the complement system, acute-phase proteins and defense collagens, cytokines function as intercellular signals and inflammatory mediators. Cytokines are produced by various cell types and have a wide range of activities, mostly dependent on their target cell[173,209,229]. They are involved in promoting an inflammatory response, cell differentiation, proliferation, or cell movement and are responsible for the interaction of the

innate immune system with the adaptive immune response. Cytokines such as interferon-γ (IFN-γ), interleukin-6 (IL-6), interleukin-1β (IL-1β), or tumor necrosis factor α (TNF-α) are pro-inflammatory signals that activate inflammation, e.g. in response to *S. aureus* infection[230–232]. Although the induction of pro-inflammatory cytokines is protective, an overshooting production or hyperinflammation can be harmful, leading to multiorgan failure, hypotensive shock, and death[233]. For the regeneration and repair of damaged tissue after the pathogen elimination, the inhibition and deactivation of inflammation is essential. Interleukin-10 (IL-10) or transforming growth factor β (TGF-β) for example are such anti-inflammatory mediators which can deactivate macrophages[209,232,234,235].

Another example of humoral mediators is the complement system that comprises a cascade of proteins activating one another in a series and resulting in direct lysis of the pathogen through pore formation, opsonization, or immune modulation[236,237]. The complement system consists of more than 30 plasma and cell-surface proteins and can be activated through three different pathways, all of which are triggered by *S. aureus*: the classical, lectin or alternative pathway[77,173,238–240]. The complement cascade, independent of the activation pathway, leading to the cleavage of complement factor 3 (C3) into C3a, that is, similar to C5a, a potent anaphylatoxin, with strong pro-inflammatory effects and capable of modulating innate immune cell recruitment[241–243]. As for cytokines, a functional complement system is essential to control the infection with pathogens like *S. aureus*[244,245]. However, dysregulation including deficiencies of complement proteins or excessive activation can lead to severe diseases such as lupus[246], multiple sclerosis[247], asthma[248], Alzheimer's[249] or sepsis[250], harming the host itself.

1.3 Pathogenic strategies of *S. aureus* to circumvent the host innate immune response

For the successful survival of *S. aureus*, the pathogen needs to escape the host defense mechanisms. To achieve this, *S. aureus* has evolved numerous immune-modulating effectors to counteract the host immune defenses by either stopping or delaying the host attack to create a time window where the bacteria have the chance to divide and/or by creating a microenvironment that facilitates better survival[251]. In the following, exemplary mechanisms and factors for different staphylococcal innate immune evasion strategies are presented.

Evasion of defensins

Defensins are one of the first defense mechanisms of innate immunity in all living species. They exert potent antimicrobial effects against an array of pathogens including bacteria and fungi and are found in large amounts in the granules of phagocytes[252]. They have been classified as α-, β and θ-defensins based on structural differences[253] and kill microorganisms by forming pores in the bacterial cytoplasmic membrane[252]. *S. aureus* has evolved mechanism to resist the antimicrobial effect of host defensins. Thus, *S. aureus* secretes staphylokinase (SAK), which has been shown to bind *α*-defensins and thereby inhibit their bactericidal effects, usually resulting in disruption of the bacterial cell membrane[254]. Another potent anti-staphylococcal peptide is the human cathelicidin LL-37, that can be cleaved by the major *S. aureus* metalloproteinase aureolysin at multiple sites leading to its inactivation in a concentration- and time-dependent manner[255,256]. Furthermore, the expression of peptidoglycan O-acetyltransferase OatA renders *S. aureus* completely resistant to lysozyme, an antimicrobial glycoside hydrolase[257]. Lysozyme is an important antimicrobial enzyme found in neutrophil granules and it is also secreted by macrophages[258]. Lysozyme disrupt the bacterial cell wall by hydrolyzing the β-1,4-glycosidic linkages between *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG)[259]. *S. aureus* uses OatA to acetylate its cell wall and thereby inhibits the binding of lysozyme[257].

Evasion of phagocytosis

S. aureus have evolved numerous survival strategies to avoid phagocytic killing which can be group in four groups: (1) interfering with the recruitment of phagocytic cells to the site of infection; (2) damaging or destroying phagocytic cells; (3) preventing its uptake by phagocytic cells and (4) avoiding the killing machinery after been phagocytosed[173].

(1) Interfering with the recruitment of phagocytic cells to the site of infection

Recruitment of phagocytic cells at the site of infection is a crucial defense process of the in innate immune response for pathogen elimination. *S. aureus* produces several virulence factors that interfere with the recruitment of phagocytic cells. Among them, the extracellular adherence protein Eap has been found to inhibit chemotaxis of leukocytes by directly interacting with the host adhesive proteins fibrinogen, vitronectin, or the intercellular adhesion molecule 1 (ICAM-1)[87,260,261]. By binding to ICAM-I, Eap prevents the binding of leukocytes to endothelial cells and inhibits the extravasation of these cells from the bloodstream into the site of infection[262]. The chemotaxis inhibitory protein of *S. aureus* (CHIPS) blocks the response of neutrophils and macrophages to the complement factor C5a and formylated peptides by binding to C5a receptor and to the formyl peptide receptor, respectively[263,264]. It is speculated that for the inhibition of neutrophil recruitment, Eap and CHIPS act together[265]. The *S. aureus* secreted staphopain A has been shown to cleave the N-terminus of the chemokine receptor CXCR2 in neutrophils, which is critical for neutrophil chemotaxis[266]. The staphylococcal superantigen-like 5 (SSL5) protein inhibits neutrophil rolling on endothelial cells by directly binding to P-selectin glycoprotein ligand 1 (PSGL-1) on neutrophils[267].

(2) Damaging or destroying phagocytic cells

S. aureus produces several exotoxins capable of damaging the plasma membrane of a wide variety of immune cells such as, neutrophils, monocytes and macrophages[97]. Thus, the pore-forming toxin α-hemolysin has been shown to induce cell death of human monocytes[100]. Also β-hemolysin has been shown to lyse monocytes[268] and neutrophils[269]. In contrast to pore-forming toxins, β-hemolysin is a sphingomyelinase that does not form pores on target cells but causes cell membrane damage by hydrolyzing sphingomyelin[268]. The bi-component toxins including γ-hemolysin (HlgAB and HlgCB), leukocidin E/D, leukocidin M/F′ and Panton-Valentine leukocidin (PVL) are highly cytotoxic towards human neutrophils and macrophages by forming pores on the cell membrane resulting in osmotic imbalance[270].

(3) Preventing the uptake by phagocytic cells

Phagocytosis is initiated by the binding of pathogens to the surface of phagocytic cells via PAMPs. However, bacteria opsonization by immunoglobulins or complement results in much more efficient phagocytic uptake. Immunoglobulins and complement are the major opsonization factors. They bind the bacterial surface and are recognized by Fc receptors (FcRs) and complement receptors, respectively, which initiate the phagocytosis process. *S. aureus*

employs numerous strategies to disrupt phagocytosis including the production of a capsule polysaccharide that conceals the bacterial surface determinants from recognition by phagocytic cells[271]. Protein A secreted by *S. aureus* binds the constant Fc region of immunoglobulin IgG and thereby prevents the engagement of Fc receptors on the surface of phagocytic cells by opsonized bacteria[272,273]. An additional protein produced by *S. aureus* that prevents phagocytosis by binding IgG is Sbi[274,275]. Staphylococcal superantigens such as staphylococcal superantigen-like 7 (SSL7) which binds to human IgA inhibits FcαR binding on phagocytic cells[276], and superantigen-like protein SSL10 that binds to IgG[277] have also been shown to confer protection from phagocytosis. *S. aureus* has evolved highly diverse strategies to inhibit the complement system. For example, the extracellular fibrinogen binding molecule (Efb) blocks the classical complement activation-dependent opsonization by binding the complement component C3 and prevent its cleavage[278]. Efb can also bind to the C3 cleavage product C3b deposited on the bacterial surface and block bacteria phagocytosis[279]. Similarly, staphylokinase mediates bacteria complement evasion by binding plasminogen and converting the bound plasminogen into its active form plasmin resulting in degradation of C3b and IgG at the bacterial surface[280]. One of the most efficient staphylococcal inhibitors of the complement system is the staphylococcal complement inhibitor (SCIN), which inhibits C3 convertases activity and thereby opsonization of *S. aureus* by C3b product[280]. Complement-dependent phagocytosis by neutrophils is also blocked by the aforementioned metalloprotease aureolysin by inhibiting the opsonization of the bacteria with C3b and the release of the chemoattractant C5a[281].

(4) Avoiding the killing machinery after being phagocytosed

Following phagocytosis by host cells, *S. aureus* possesses sophisticated strategies to resist potent bactericidal mechanisms either by resisting phagolysosomal killing, or by escaping from the phagosome into the cytoplasm. *S. aureus* initially resides in phagosomes within phagocytic cells. In this regard, it has been reported that survival of *S. aureus* within macrophages was associated with a failure of phagosomal maturation and acidification[282]. *S. aureus* produces multiple factors such as carotenoid staphyloxanthin, catalase, superoxide dismutase, and peroxiredoxins to neutralize the killing effect of oxygen species within the phagolysosome[283–285]. Furthermore, *S. aureus* induces modifications in the cell walls that increases resistance to lysozyme, one of the most abundant components found in phagolysosomes, through O-acetylation of peptidoglycan by OatA[257].

Besides the studies reporting the capacity of *S. aureus* to survive and replicate within the phagosome[286], other studies however have revealed the capacity *of S. aureus* to escape from the phagosome into the cytoplasm of the phagocytic cells[145,287]. *S. aureus* phagosome escape

has been connected to the production of α-PSMs [145] or Hla[287], β-PSMs, Hld and Hlb[288,289]. The intracellular niche is not only conferring protection from the immune system or antibiotics but is seems to contribute to *S. aureus* dissemination within the host in accord with a 'Trojan horse" mechanism[290–292].

An additional strategy for *S. aureus* to overcome the killing mechanisms of phagocytic cells is to induce different cell death pathways, including mainly apoptotic host cell death[293–295], but also necrosis and pyronecrosis[296].

1.4 Experimental approaches to study host/pathogen interactions

1.4.1 Simultaneous transcriptional profiling of host and pathogen

In infection biology, understanding the host-pathogen interplay is of great interest as it can help to design new therapeutic interventions against important pathogens such as *S. aureus*. The simultaneous transcriptional profiling of the host and the pathogen can uncover the impact of both organisms on each other and identify new virulence factors and/or host cell pathways expressed during the infection process. Although it is technically challenging, several methods have evolved over the last decades to make it feasible.

The microarray technology introduced in the 1990s was based on immobilized DNA probes hybridized to fluorescence labeled cDNA and enabled the analysis of relative transcript abundances by measuring fluorescence signal intensities[297,298]. However, this method was restricted to known or predicted mRNAs. The development of high -resolution tilling arrays had overcome these limitations and enabled the gene expression profiling of the whole genome of an organism[299–302]. Nevertheless, also tilling arrays had great drawbacks, including for instance a limited dynamic range with an upper (saturation) and a lower limit (sensitivity), high costs, especially for large genomes, and problems with cross-hybridization[303–305].

With RNA-sequencing (RNA-seq) technologies based on next-generation sequencing (NGS) platforms, such as Illumina IG[306,307] or Roche 454 Life Science[308,309] systems, the above mentioned limitations had been overcome and a new era in transcriptomics started. RNA-seq is a digital quantification method based on massive parallel sequencing of cDNA libraries that have been reverse transcribed from RNA molecules, followed by mapping of the obtained reads onto a reference genome[304]. By using special protocols for library preparation, even the information about transcript directionality can be obtained, especially relevant for the analysis of dense genomes and active antisense transcription[310–312]. Other advantages are the extreme sensitivity, the accurate determination of borders, alternative splicing, and processing events through the digital quantification used in RNA-seq approaches[304]. Using RNA-Seq, the host transcriptional response to *S. aureus* infections[313] as well as the response of *S. aureus* to different stressors[314–316] have been studied. However, an experimental setup where the transcriptomes of interacting pathogens with their host are simultaneously profiled provides a new and unperturbed view of the infection process and the interaction of both partners (Fig. 2). This approach, called dual RNA-seq, holds some major challenges, e.g. differences in total RNA content between eukaryotic and pathogenic cells, depletion of rRNA species before sequencing, and RNA degradation[303,304]. Depending on the experimental design, protocols have been established to overcome some of these problems, including selective rRNA depletion kits

or RNA stabilization solutions[317,318]. Besides the challenges before sequencing, there are also difficulties after receiving the raw reads since the massive amount of data generated through RNA-seq needs to be stored and processed, including bioinformatics tools[303,319,320]. Nevertheless, the ongoing improvement of RNA-seq technology together with the precious insights into infection processes probably will make dual RNA-seq approaches the gold standard for transcriptomic studies in infection biology[304]. Moreover, single-cell RNA sequencing technologies have already been achieved for eukaryotic cells[321–325], and single-cell dual RNA-seq become more and more feasible to resolve the highly diverse response of individual host cells as well as bacterial cells within a population[304].

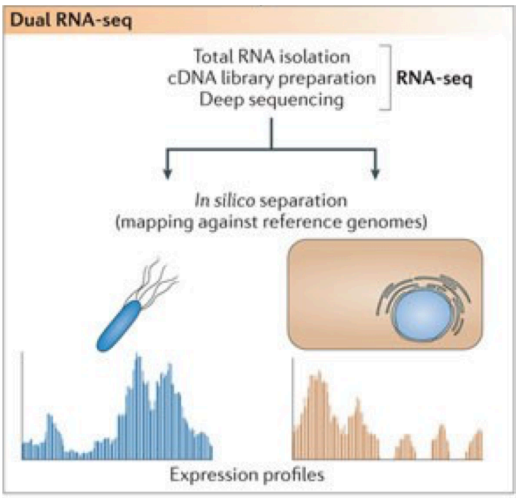

Figure 2 | Schematic concept of dual RNA-seq.
Scheme of the concept of dual RNA-seq, where total RNA isolation, cDNA library preparation and deep sequencing is followed by the *in silico* separation of the generated host and pathogen sequencing reads by mapping to the respective reference genome. Reprinted by permission from Macmillan Publishers Ltd: *Nature Review Microbiology* Westermann *et al.*, copyright 2012[304].

1.4.2 *S. aureus* pKikume

In this study, a *S. aureus* strain carrying the mKikumeGR proliferation reporter system that allows to investigate the metabolic and proliferation status of intracellular bacteria generated by Seiß *et al.*[326] has been used. This reporter system consists of a monomeric photoswitchable fluorescent protein emitting green fluorescence in its original state and can be photoswitched to emit red fluorescence by UV or violet light[327]. The monomeric form of this protein is based on the KikGR fluorescence protein reported by Tsutsui *et al.* and originates from the stony coral *Favia favus*[328]. Photoconversion is performed by a 405 nm light pulse switching green mKikumeGR to red mKikumeGR, measured with 475 nm and 555 nm excitation, respectively. Crucial in this proliferation reporter system is that the

photoconversion of the fluorescence signal is irreversible (Fig. 3). In the course of intracellular pathogen proliferation, the red fluorescent protein after photoconversion is progressively diluted among daughter cells, and green fluorescent proteins can only be synthesized *de novo* if the intracellular pathogen is metabolically active[329]. With this feature, one can closely correlate the metabolic activity and/or proliferation of intracellular bacteria with the recovery of green fluorescence after photoconversion.

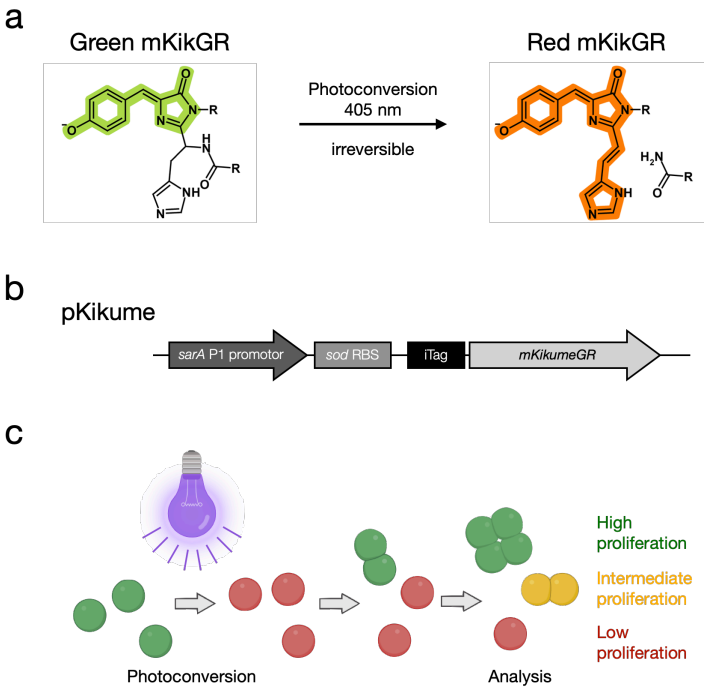

Figure 3 | mKikumeGR: structure, plasmid construct and principle of the reporter system.
(a) Chemical structure of green and red mKikumeGR. (b) Schematic vector construct for pGL485 used for mKikume expression in *S. aureus* SH1000. (c) Schematic overview of the mKikumeGR reporter system principle (created with BioRender.com).

Photoswitchable fluorescence proteins are of great interest since the controlled conversion of fluorescence proteins enables to mark and track them within cells[330,331]. Especially super-resolution microscopy techniques such as STORM or PALM, combined with photoswitchable proteins are promising systems to localize the individual fluorescent signals with high accuracy[332–335]. As the molecular dynamics inside a cell can be quite fast, the efficient and rapid conversion of the fluorescence protein, as well as bright fluorescence signals, are necessary for successful application of these tecniques[327].

2 AIMS OF THIS STUDY

S. aureus is an opportunistic human pathogen and the causative agent of a variety of infections. Unfortunately, treatment failure is a growing problem of *S. aureus* infections that is likely due to the combined effects of antibiotic resistance and recurrent infections caused by this pathogen. Although *S. aureus* is generally considered an extracellular pathogen, it is capable to invade and survive, even for long periods, within host cells including professional phagocytes such as macrophages as well as non-professional phagocytes such as epithelial cells. The intracellular niche facilitates *S. aureus* persistence and/or proliferation and can also be used as a "Trojan horse" for dissemination within the host and infection of distant sites. Therefore, there is a clear need for novel therapeutic approaches that can lead to the eradication of intracellular *S. aureus*. To develop these novel approaches, it is necessary to achieve a better understanding of the survival mechanisms and bacterial virulence factors used by *S. aureus* to survive in the intracellular milieu. Hence, the overall aim of this study was to investigate the strategies used by *S. aureus* to survive and multiply within professional phagocytes (macrophages) and non-professional phagocytes (epithelial cells) as well as the influence of the internalization pathway of *S. aureus* into the host cell on the fate of internalized bacteria. For this purpose, an infection model was established using a *S. aureus* strain carrying the proliferation reporter mKikumeGR that enable to monitor the metabolic activity and proliferation status of the internalized bacteria. To achieve these aims, the following steps were taken:

(I) Establishment of an *in vitro* infection model of macrophages and epithelial cells using *S. aureus* pKikume and optimization of the photoconversion procedure for the reporter fluorescence protein mKikume.

(II) Development and optimization of a protocol for RNA extraction and processing from *S. aureus*-infected macrophages after FACS sorting of subpopulations according to the fluorescence of the bacteria that they harbor (mainly green or red).

(III) Compilation of bioinformatic and statistic tools to pre-process and simultaneously analyze the host and pathogen transcriptome datasets generated by next-generation deep sequencing technology (dual RNA-seq approach).

(IV) Validation of the dual RNA-seq approach by qRT-PCR on a selected gene set.

(V) Determination of the impact of the *S. aureus* internalization route on the bacterial metabolic activity and survival/proliferation within different host cell types by counting viable intracellular bacteria after plating, flow cytometry analysis and different microscopy techniques.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacterial strains

Table 1 | Bacterial strains used in this study.

***S. aureus* strain**	**Source**	**Reference**
SH1000 wild type	Kindly provided by Foster, S.; Sheffield, UK	[336]
SH1000 pKikume	Kindly provided by Müller, A.; Magdeburg, Germany	[326]
SH1000 Δhla	Kindly provided by Foster, S.; Sheffield, UK	[337]

3.1.2 Mouse strains

Table 2 | Mouse strain used in this study.

Mice strain	**Description**
C57BL/6 WT	Harlan-Winkelmann (Borchen, Germany)

3.1.3 Cell lines

Table 3 | Cell lines used in this study.

Cell line	**Source**	**Reference**
Immortalized macrophages (IMΦ)	Kindly provided by Brinkmann, M. M.; HZI Braunschweig, Germany	[338]
HEp-2 (ATCC® CCL-23™)	Sigma Aldrich, Munich, Germany	[339]
HC-11 (ATCC® CRL-3062™)	DSMZ, Braunschweig, Germany	[340]

3.1.4 Chemicals and Kits

Table 4 | Chemicals, kits and their suppliers used in this study.

Chemical/kit	**Supplier**
10% Mini-PROTEAN® TGX™ Precast Protein Gel	Bio-Rad, Hercules, CA, USA
4x Laemmli Sample Buffer	Bio-Rad, Hercules, CA, USA
Acetic acid (100%) ($C_2H_4O_2$)	Carl Roth, Karlsruhe, Germany
Amersham ECL detection reagent	GE Healthcare, Boston, MA, USA

Chemical/kit	Supplier
BD Columbia Agar with % Sheep Blood	BD Pharmingen, Heidelberg, Germany
Brain Heart Infusion Broth	Carl Roth, Karlsruhe, Germany
CytoTox 96® Non-Radioactive Cytotoxicity Assay	Promega, Madison, WI, USA
Dextran, Alexa Fluor™ 647; 10,000 MW	Invitrogen, Carlsbad, CA, USA
eBioscience Intracellular Fixation & Permabilization Buffer Set	LIFE Technologies, Carlsbad, CA, USA
EDTA	Livchem, Frankfurt am Main, Germany
Ethanol absolute	AppliChem, Darmstadt, Germany
Filipin III, ready made solution	Sigma Aldrich, Munich, Germany
GLY-ARG-GLY-ASP-SER-PRO (GRGDSP), RGD peptide	Sigma Aldrich, Munich, Germany
Glycin	Sigma Aldrich, Munich, Germany
Lysing matrix B	MP Biomedicals, Solon, OH, USA
Lysostaphin	Sigma Aldrich, Munich, Germany
Methanol, ≥ 99.9 %	Sigma Aldrich, Munich, Germany
Methyl-beta-cyclodextrin, cell culture tested	Sigma Aldrich, Munich, Germany
NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina®	New England Biolabs, Ipswich, MA, USA
Nuclease-free water	Qiagen, Hilden, Germany
Potassium chloride (KCl)	Merck, Darmstadt, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Merck, Darmstadt, Germany
PureLink™ DNase Set	Invitrogen, Carlsbad, CA, USA
PureLink™ RNA Mini-Kit	Invitrogen, Carlsbad, CA, USA
RiboGuard Rnase Inhibitor 40 U/µL	Epicenter, Madison, WI, USA
Ribo-off® rRNA Depletion Kit (Bacteria)	Vazyme Biotech, Nanjing, China
Ribo-off® rRNA Depletion Kit (Human/Mouse/Rat)	Vazyme Biotech, Nanjing, China
RNaseZap	Ambion, Austin, TX, USA
SensiFast SYBR No-ROX One-Step kit	Bioline, Luckenwalde, Germany
SER-ASP-GLY-ARG-GLY, RGD reverse peptide	Sigma Aldrich, Munich, Germany

Chemical/kit	Supplier
Skim milk powder	Sigma Aldrich, Munich, Germany
Sodium chloride (NaCl)	Merck, Darmstadt, Germany
SDS (Sodium Dodecyl Sulfate)	Bio-Rad, Hercules, CA, USA
Sodium hydroxide (NaOH)	Carl Roth, Karlsruhe, Germany
Sodium phosphate dibasic dihydrate ($Na_2HPO_4 \cdot 2H_2O$)	Sigma Aldrich, Munich, Germany
Triton X-100	Sigma Aldrich, Munich, Germany
Trizma Base	Sigma Aldrich, Munich, Germany
TRIzol™ Reagent	Invitrogen, Carlsbad, CA, USA
Trypan blue	Sigma Aldrich, Munich, Germany
Tween20	Carl Roth, Karlsruhe, Germany

3.1.5 Antibodies, enzymes and sera

Table 5 | Antibodies, sera and their suppliers used in this study.

Antibody/Serum	Supplier
Anti-mouse CD16/CD32	BD Pharmingen, Heidelberg, Germany
Anti-staphylococcal α-toxin	Sigma Aldrich, Munich, Germany
APC anti-mouse/human CD11b	Biolegend, San Diego, CA, USA
Anti-Arginase 1 Monoclonal Antibody (A1exF5), PE-Cy7	LIFE technologies, Carlsbad, CA, USA
Fetal Calf Serum (FCS)	Gibco, Carlsbad, CA, USA
Anti-mouse F4/80-FITC	Biolegend, San Diego, CA, USA
Goat-anti-rabbit Ig-HRP	Imgenex Corporation, San Diego, CA, USA
Anti-Integrin β1	Cell Signaling Technology, Danvas, MA, USA
Anti-mouse CD11c-PE	Biolegend, San Diego, CA, USA
Anti-mouse CD206-PE	Biolegend, San Diego, CA, USA
Rabbit IgG Isotype control Antibody	Invitrogen, Carlsbad, CA, USA

3.1.6 Media and buffers

Table 6 | General media and buffers used in this study.

Buffer/medium	Amount of chemical/solution
0.5 M EDTA, pH 8.0 (1 L)	186.12 g EDTA $Na_2 \cdot 2H_2O$ *ad* 1 L H_2O *dest.* adjust pH 8.0 with NaOH
1 x PBS, pH 7.4 (1 L)	50 mL 20 x PBS, pH 7.4 *ad* 1 L H_2O *dest.*, autoclaved
1 x TAE, pH 8.5 (1 L)	20 mL 20 x TAE, pH 8.4 *ad* 1 L H_2O *dest.*, autoclaved
1 x Transfer buffer	100 mL 10 x Transfer buffer 200 mL Methanol *ad* 1 L H_2O *dest.*
1 M Tris, pH 8.8	118.2 g Tris-HCl *ad* 500 mL H_2O *dest.* adjust pH 8.8 with NaOH, filtered
10 x Running buffer	60 g Trizma base 288 g Glycine 20 g SDS *ad* 2 L H_2O *dest.* dilute to 1 x for running SDS-PAGE gels
10 x Transfer buffer	60 g Trizma base 288 g Glycine *ad* 2 L H_2O *dest.*
20 x PBS, pH 7.4 (1 L)	160 g NaCl 4 g KCl 15.2 g $Na_2HPO_4 \cdot 2H_2O$ 4 g KH_2PO_4 *ad* 1 L H_2O *dest.* adjust pH 7.4 with NaOH

50 x TAE, pH 8.5 (1 L)	242 g Trizma Base 57.1 mL Acetic acid 100 mL 0.5 EDTA, pH 8.0 *ad* 1 L H_2O *dest*. adjust pH 8.5 with NaOH
BHI broth (1 L)	37 g Brain-Heart-Infusion Broth *ad* 1 L H_2O *dest*., autoclaved

3.1.7 Cell culture solutions

Table 7 | Cell culture solutions used in this study.

Solution	**Supplier**
Dulbecco's Modified Eagle Medium (DMEM)	LIFE technologies, Carlsbad, CA, USA
Roswell Park Memorial Institute (RPMI) 1640 medium	LIFE technologies, Carlsbad, CA, USA
Trypsin-EDTA (0.05%)	LIFE technologies, Carlsbad, CA, USA

3.1.8 Growth factors and antibiotics

Table 8| Growth factors, antibiotics and their suppliers used in this study.

Growth factor/antibiotic	**Supplier**
Antibiotic/Antimycotic (100X)	Thermo Scientific, Bonn, Germany
Epidermal growth factor	Sigma Aldrich, Munich, Germany
Gentamicin	Gibco, Carlsbad, CA, USA
Insulin solution from Bovine pancreas	Sigma Aldrich, Munich, Germany
LPS from *E. coli* O111:B4, ready-made solution	Sigma Aldrich, Munich, Germany
Recombinant mouse M-CSF (carrier free)	Biolegend, San Diego, CA, USA
Recombinant mouse IL-13 (carrier free)	Biolegend, San Diego, CA, USA
Recombinant mouse IL-4 (carrier free)	Biolegend, San Diego, CA, USA

3.1.9 Primer

Table 9 | Oligonucleotide primer used in this study.

Target	Forward/ Reverse	Sequence (5'-3')	Annealing temperature
mm_βactin	for	TAA AAC GCA GCT CAG TAA CAG TCC G	58-60 °C
	rev	TGG AAT CCT GTG GCA TCC ATG AAA C	
mm_IL-1β	for	CAC AGC AGC ACA TCA ACA AG	58 °C
	rev	GTG CTC ATG TCC TCA TCC TG	
mm_IL-6	for	CTG GTG ACA ACC ACG GCC TTC CCT A	58 °C
	rev	ATG CTT AGG CAT AAC GCA CTA GGT T	
mm_Arginase-1	for	CAG AAG AAT GGA AGA GTC AG	58 °C
	rev	CAG ATA TGC AGG GAG TCA CC	
mm_CD69	for	AGG CTT GTA CGA GAA GTT GGA	58 °C
	rev	AGT TCA CCA GAA TAT CGC TTC AG	
mm_PPARɣ	for	TGT GGG GAT AAA GCA TCA GGC	58 °C
	rev	CCG GCA GTT AAG ATC ACA CCT AT	
mm_TNF-α	for	CAT CTT CTC AAA ATT CGA GTG ACA A	58 °C
	rev	TGG GAG TAG ACA AGG TAC AAC CC	
sao_16S rRNA	for	ACG GTC GCA AGA CTG AAA CT	60 °C
	rev	TAA GGT TCT TCG CGT TGC TT	
sao_AgrA	for	AAC TGC ACA TAC ACG CTT ACA	60 °C
	rev	GGC AAT GAG TCT GTG AGA TTT	
sao_MepA	for	GCG AGA GGT GAA ACG TTA GC	60 °C
	rev	CTG ATT GCA GTA CCC AAA GC	
sao_Psmβ2	for	GGC ACA AGC ATT GTG GCA T	60 °C
	rev	ATT GCT TCT GCT AGT CCA GTC ATT T	
sao_Hla	for	GGC CTT ATT GGT GCA AAT GT	60 °C
	rev	AGC GAA GTC TGG TGA AAA CC	
sao_KatA	for	TGT AGC AGG AGA ACG TGG TG	60 °C
	rev	CCG TCC AGA AAT CCC AGT TA	

Target	Forward/ Reverse	Sequence (5'-3')	Annealing temperature
sao_LrgB	for	ACA ATC TGT TTT GCG ATT CCG	60 °C
	rev	CTG TAG TTG CTG CTT GAG GT	
sao_Pbp1	for	AGG TAG CGG TTT TGT GTC C	60 °C
	rev	TAT CCT TGT CAG TTT TAC TGT C	
sao_Gmk	for	CGT TTT ATC AGG ACC ATC TGG A	60 °C
	rev	CTA CGC CAT CAA CTT CAC CTT C	
sao_Map	for	AGG ACA AAC TAC CGG CAA CTC	60 °C
	rev	TGC CAT CTT TCC AAG TAA TCG	
sao_FnbpA	for	ACA AGT TGA AGT GGC ACA GCC	60 °C
	rev	CCG CTA CAT CTG CTG ATC TTG TC	
sao_ClfA	for	ATG TGA CAG TTG GTA TTG ACT CTG G	60 °C
	rev	TAG GCA CTG AAA AAC CAT AAT TCA GT	

3.1.10 Devices and consumables

Table 10 | Devices, consumables and their suppliers used in this study.

Device/consumable	Supplier
10 x 10 LED array built of UV 405 nm LED's	Strato, Berlin, Germany
96-well micro-titer plates	Sarstedt, Nümbrecht, Germany
Autoclave	Belimed AG, Zug, Switzerland
Basic Meter PB-11	Sartorius AG, Göttingen, Germany
BD™ LSR II SORP Flow cytometer	BD Bioscience, Franklin Lakes, USA
BD™ FACS ARIA II SORP Flow cytometer	BD Bioscience, Franklin Lakes, USA
Blotting chamber Mini Trans-Blot cell	Bio-Rad, Hercules, CA, USA
Cannula	B. Braun, Melsungen, Germany
Cell culture plates (4, 6, 12, 24 wells)	Sarstedt, Nümbrecht, Germany
Cell strainer	Sigma Aldrich, Munich, Germany
Centrifuge 5810 R	Eppendorf AG, Hamburg, Germany
Eppendorf Research® plus pipettes	Eppendorf AG, Hamburg, Germany
Falcon Tube (15 mL, 50 mL)	Sarstedt, Nümbrecht, Germany
Forma Direct Heat CO_2 Incubator	Thermo Fisher Scientific Inc., Waltham, USA

Forma Scientific CO_2 Water Jacketed Incubator	Thermo Fisher Scientific Inc., Waltham, USA
Gloves (latex, nitrile)	Shield-Scientific, Bennekom, Netherlands
Heraeus Biofuge Pico	Heraeus Holding GmbH, Hanau, Germany
Heraeus Megafuge 1.0	Heraeus Holding GmbH, Hanau, Germany
HeraSafe KS Safety Cabinet Class II	Heraeus Holding GmbH, Hanau, Germany
Heratherm Compact microbiological Incubator	Thermo Fisher Scientific Inc., Waltham, USA
ID03 Invertoscope Inverted Microscope	Carl Zeiss AG, Oberkochen, Germany
Lab Combination Freezer LCv 4010 MediLine	Liebherr, Bulle, Schweiz
Laboratory Freezer LGT 4725	Liebherr, Bulle, Schweiz
Laboratory Freezer Premium	Liebherr, Bulle, Schweiz
Microtiter plate	Nalge Nunc International, Rochester, NY, USA
NanoDrop One Spectrophotometer	Thermo Fisher Scientific Inc., Waltham, USA
Nitrocellulose membrane	GE Healthcare, Boston, MA, USA
Novaspec II Visible Spectrophotometer	Pharmacia Biotech, Uppsala, Sweden
Petri dish	Sarstedt, Nümbrecht, Germany
Phasemaker™ Tubes	Invitrogen, Carlsbad, CA, USA
Pipette tips	Eppendorf AG, Hamburg, Germany
Reaction vessels (0.2 µL, 1.5 µL, 2.0 µL)	Eppendorf AG, Hamburg, Germany
Safe 2020 Class II Biological Safety Cabinet	Thermo Fisher Scientific Inc., Waltham, USA
SUNRISE Absorbance Reader	Tecan Trading AG, Männedorf, Switzerland
Syringe (1 mL, 5 mL, 20 mL)	B. Braun, Melsungen, Germany
Thermomixer comfort	Eppendorf AG, Hamburg, Germany
Vertical Electrophoresis Cell Mini-PROTEAN	Bio-Rad, Hercules, CA, USA
Vortex VF2	IKA®-Werke GmbH & CO. KG, Staufen, Germany
Water bath 1003	GFL Gesellschaft für Labortechnik GmbH, Burgwedel, Germany

3.2 Methods

3.2.1 Bacterial culture

The *S. aureus* strains used in this study were strain SH1000 wild type (WT), the mKikumeGR reporter plasmid carrying strain SH1000 pKikume and the alpha-toxin deficient (Δhla) isogenic *S. aureus* mutant strain. Staphylococci were grown in brain-heart-infusion medium (BHI) overnight with steady shaking (150 rpm) at 37°C. The next day, bacterial cultures were refreshed at a ratio of 1:1000 (V/V) in BHI medium, grown to an optical density of 0.3-0.5 at OD_{600} (OD_{600}) and collected by centrifugation for 10 min at 16,000 × g and 4°C. After washing with sterile PBS, the bacterial cultures were diluted to the required concentrations in cell culture medium. The number of viable bacteria was determined after serial plating on 5% sheep blood agar followed by incubation for 24h at 37°C and calculated as bacterial CFU per ml.

3.2.2 Photoconversion of mKikumeGR

Photoconversion of intracellular *S. aureus* pKikume from green to red fluorescence was performed by a pulse of violet light at 405 nm wavelength using a 10×10 LED array (half-viewing angle: 15°; Radiant Power: 10 mW). A 90 sec light pulse was found to be optimal for switching the fluorescence without damaging the host cells. The photoconversion of intracellular bacteria was performed 30 min before sampling at different time points.

3.2.3 Preparation and culture of eukaryotic cells

3.2.3.1 Preparation and culture of primary bone marrow derived macrophages (BMDM's)

Mouse bone marrow derived macrophages were differentiated from extruded bone marrow of femur and tibia of female C57BL/6 wild-type mice by flushing the bones with cold 1 x PBS supplemented with 2% heat-inactivated fetal calf serum (h.i. FCS) by using a 23G needle. The cell suspension was passed through a 70µm cell strainer and treated with NH_4CL for 10 min on ice to remove red blood cells. After centrifugation at 500 x g for 5 min at 4°C and discarding the supernatant the pelleted cells were resuspended in DMEM supplemented with 10% h.i. FCS and cultured in petri dishes in the presence of 50 ng/ml M-CSF and 1 x Antibiotic/Antimycotic (AB/AM) for one week at 37°C and 5% CO_2. Medium change was performed at day 3 of culture.

3.2.3.2 Induction of M1 and M2 macrophages

Mature BMDM's were polarized into M1 or M2 macrophages 7 days after isolation from bone marrow using 100ng/ml LPS for M1 activation and 10ng/ml IL-4 and 10ng/ml IL-13 for M2 activation supplemented to DMEM 10% h.i. FCS and 1 x AB/AM. Both macrophage subtypes were adjusted to a cell density of 5 x 10^5 cells/ml, seeded in multi-well cell culture plates and activated for 24h in the presence of the mentioned factors for M1 or M2 polarization. Polarized macrophages were then detached using 0.05% trypsin and used for further experiments or analysis.

3.2.3.3 Culture of immortalized macrophages (IMΦ's)

Immortalized mouse macrophages were cultured in DMEM supplemented with 10 % h.i. FCS and 1 x AB/AM at 37°C and 5% CO_2 in cell culture dishes. Cells were subcultured into fresh medium every 3-4 days after detaching them using a cell scraper.

3.2.3.4 Culture of human (HEp-2) and murine (HC-11) epithelial cells

The human epithelial cell line HEp-2 (ATCC CCL23) was cultured in DMEM supplemented with 10 % h.i. FCS at 37°C in 5% CO_2 in cell culture flasks. Mouse epithelial HC-11 cells (ATCC CRL-3062) were grown in RPMI 1640 supplemented with 10% h.i. FCS, 5 µg/ml bovine insulin, 10 ng/ml EGF and 4 mM L-glutamine under the same conditions as HEp-2 cells. Both cell lines were subcultured every 2-3 days by using 0.05% trypsin for detaching.

3.2.4 Cytotoxicity Assay

To measure the potential cytotoxic effect of the UV pulse of 405nm used for photoconversion on macrophages, a lactate dehydrogenase (LDH)-assay was performed using a CytoTox 96 Non-Radioactive Cytotoxicity Kit according to the manufacturer's recommendations (Promega). With this colorimetric assay the amount of LDH released into the culture supernatant by UV-treated and untreated control macrophages was measured and cell death was calculated as following:

$$LDH\ release\ [\%] = 100 \times \left(\frac{LDH\ release\ in\ test\ sample - spontaneous\ LDH\ release}{maximum\ LDH\ release - spontaneous\ LDH\ release}\right)$$

The maximum release was achieved by adding 1% (V/V) Triton X-100 to untreated macrophages.

3.2.5 *In vitro* Infection Assays

One day prior to infection, cultured cells were harvested either by scratching or by trypsin treatment, seeded at a density of 5×10^5 cells/ml in multi-well cell culture plates and incubated overnight (o/n) at 37°C and 5% CO_2 in the corresponding cell culture medium without AB/AM supplementation. The following day, cells were infected with *S. aureus* at a multiplicity of infection (MOI) of 5:1. After 2h of infection, the culture medium was replaced by medium containing 100 µg/ml gentamicin and 5 µg/ml lysostaphin to kill all extracellular remaining bacteria (gentamicin-lysostaphin protection assay).

In some experiments, the cultured cells were preincubated with specific inhibitors prior to the infection. For blocking the β1 integrin receptor, cells were incubated with either 100 µM of RGD peptide or an anti-β1-integrin antibody diluted to 1:500 for 15 min or 1 h, respectively, at 37°C in 5% CO_2. Cells treated with reverse sequence RGD peptide or with isotype control antibody were used as negative control. For the inhibition of caveolae formation, cells were incubated with 10 mM methyl-β-cyclodextrin (MβCD) or 20 µM filipin III for 1 h at 37°C in 5% CO_2. Cells were then carefully washed twice with 1 x PBS and infected with *S. aureus* as described above. The RGD peptide and the reverse sequence RGD were not removed by washing and were kept in the culture medium throughout the infection.

3.2.6 Killing assay of intracellular *S. aureus*

Cells were infected for 2h as described in 3.2.5. At different time points post infection (p.I.), cells were washed with sterile 1 x PBS, intracellular bacteria were released after lysing the host cells by adding $_{dd}H_2O$ supplemented with 0.1% Triton X-100 for 5 min at room temperature (RT), and serial dilutions of cell lysates were plated on 5% sheep blood agar. After incubation at 37°C o/n, bacterial colonies were counted, and killing rates as well as colony forming units per ml (CFU/ml) were calculated. Unless otherwise stated, the amount of viable intracellular bacteria after the 2h phagocytosis (0h p.I.) was used as reference for calculation.

3.2.7 Immunostaining

3.2.7.1 Staining of cell surface markers

To validate the successful polarization of M1 and M2 macrophages from differentiated BMDM's, cells were stained using antibodies against specific cell surface antigens and analyzed by flow cytometry. Briefly, a 200µl cell suspension of each macrophage subset was incubated with 0.25µg/ml mouse anti FcγIII/II (CD16/32) antibody for 5 min on ice to block FcγIII/II receptors and thus to reduce non-specific immunofluorescent staining. M1 and M2 macrophage suspensions were then incubated with 2µl FITC-conjugated anti-F4/80 and 2µl

APC-conjugated anti-CD11b antibodies. PE-conjugated anti-CD11c antibodies (2µl) and 2µl of PE-conjugated anti-CD206 antibodies were also added to the M1 and M2 subset, respectively. After 1.5 h incubation protected from light at 4°C, cells were washed with 3ml of the corresponding cell culture medium and centrifuged at 180 x g for 10 min at 4°C. The supernatants were discarded and cells were further stained for intracellular antigens or directly analyzed by flow cytometry.

3.2.7.2 Staining of intracellular antigens

After blocking CD16/32 and staining the surface markers F4/80 and CD11b the cells were fixed using the eBioscience™ Intracellular Fixation & Permeabilization Buffer Set from Invitrogen. Briefly, cells were fixed with 100µl of fixation buffer for 60 min at room temperature (RT) protected from light followed by a washing step where 2ml of 1 x permeabilization buffer was added and the cell suspension was centrifuged at 600 x g for 5min at RT. After another washing step the cell pellet was resuspended in 100µl 1 x permeabilization buffer, 2.5µl PE-Cy7-conjugated anti-arginase 1 antibodies were added and the cell suspension was incubated for 60min at RT protected from light. The cells were washed twice as described above and resuspended in medium for flow cytometry analysis.

3.2.7.3 Flow cytometry

After *in vitro* infection with *S. aureus* pKikume, the cells were collected at different time points p.I. with photoconversion of the intracellular bacteria 30min before sampling. Cells were then washed with 1 x PBS, trypsinated and analyzed using a flow cytometer. The mKikumeGR fluorescence protein expressed by *S. aureus* pKikume was measured at 561 nm excitation and 582/15 nm emission for the red fluorescence signal and at 488 nm excitation and 525/50 nm emission for the green fluorescence signal. Based on the fluorescence signal of the intracellular bacteria, the infected cells were physically sorted into subpopulations harboring mainly red- or green-fluorescent bacteria and further used for RNA isolation and transcriptional analysis. A minimum of 1 x 10^6 cells of each cell population was sorted using the Aria-II SORP machine to get sufficient concentrations and quality of RNA for further analysis.

For flow cytometry analysis of surface markers and intracellular antigens, cells were washed with the corresponding cell culture medium after the staining procedure and centrifuged at 180 x g for 10 min at 4°C. The supernatants were discarded and cells were resuspended in the remaining cell culture medium followed by FACS measurement.

All flow cytometry analyses were performed on a LSR II SORP and cell sorting on an Aria-II SORP. The acquired data were analyzed using the FlowJo X software. Unstained samples were used as negative controls and to setup gating strategies.

3.2.7.4 Western blot analysis

To detect secreted bacterial proteins, cell culture supernatants were collected and sterile filtrated using 0.2µm syringe filter. The samples were separated on a 10% SDS-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. Following 2h blocking in 5% (W/V) skim milk in 1 x TBS supplemented with 0.05% Tween-20 (TBST) and three washing steps in TBST for 10min with steady shaking, the membranes were incubated o/n with a primary rabbit-anti-staphylococcal alpha-toxin antibody diluted 1:1000 in 2.5% skim milk at 4°C with steady shaking. After another 3 washing steps a secondary goat-anti-rabbit IgG coupled with horseradish peroxidase and diluted 1:1000 in 2.5% skim milk was added and further incubated for 2 h at RT. After final three washing steps using TBST, the membrane was developed using the Amersham ECL detection reagents by chemiluminescence according to the manufacturer's protocol and recorded with a ChemiDoc imager.

3.2.8 Dual RNA-seq

3.2.8.1 Total RNA extraction and purification

Total RNA was isolated from *S. aureus* pKikume infected IMΦ's (described in section 3.2.5) using the PureLink™ RNA Mini-Kit (Invitrogen) according to the manufacturer's instruction. Pure bacterial culture of *S. aureus* grown in BHI to mid-log phase and uninfected macrophages were used as control samples. Prior to RNA extraction, mechanical sample disruption was performed using lysing matrix B tubes and a FastPrep-24 instrument at an intensity of 6.5 m/s for 3 × 30 sec with 1min resting on ice between the runs. Samples were subjected to PureLink™ on-column DNase treatment (Invitrogen) to remove DNA contamination according to the manufacturer's instruction. Quality and quantity of the RNA samples were determined using a NanoDrop One spectrophotometer and an Agilent 2100 Bioanalyzer. DNA-depleted RNA samples were further subjected to ribosomal RNA (rRNA) depletion using the Ribo-off rRNA depletion kit for bacteria and human/mouse/rat (Vazyme). Depletion of both eukaryotic and bacterial rRNA was performed simultaneously following the manufacturer's instruction recommended for bacteria with additional 1µl rRNA probe for human/mouse/rat added to the hybridization mix. The rRNA depletion was confirmed using an Agilent 2100 Bioanalyzer (Fig. 4).

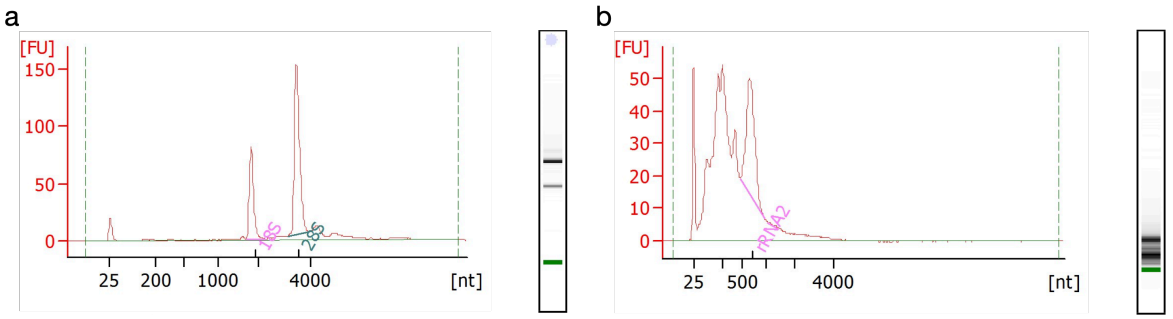

Figure 4 | Exemplary electropherogram of a sample before and after rRNA depletion.
The depletion of rRNA was assessed using the Agilent 2100 Bioanalyzer. The graph shown on the left side (a) depicts the electropherogram before rRNA depletion showing clear peaks of the 18S and 28S rRNA (RIN value = 10). The electropherogram on the right side (b) shows the sample depleted of rRNA after Ribo-Off treatment, missing the 18S and 28S peaks.

3.2.8.2 RNA-seq library preparation and sequencing

cDNA libraries were prepared from mRNA of each sample using the NEBNext Ultra II Directional RNA library Prep Kit followed by sequencing on an Illumina NovaSeq 6000 system at the technology platform Genome Analytics (GMAK) of the Helmholtz-Centre for Infection Research in Braunschweig under the direction of Dr. Robert Geffers. Parameters for sequencing were set to generate 100 million reads per sample with 150bp in lengths and paired ended. For the staphylococcal control samples only 50 million reads per sample were generated.

3.2.8.3 RNA-seq data processing

The fastq sequencing data provided by the GMAK platform were further analyzed using a bioinformatic workflow developed in this thesis that was fitted to the specific challenges of simultaneously investigating the transcriptional profile of the eukaryotic host cell and the intracellular *S. aureus*. Using the open-access quality control tool FastQC, the RNA-seq dataset was quality controlled followed by a *in silico* removal of rRNA sequencing transcripts (reads) that remained despite the rRNA depletion by Ribo-Off. After another round of quality control with FastQC, trimming of the reads in respect of sequencing quality, adapter removal and length filtering was performed using Trimmomatic. Reads were trimmed from the 3'-end if the Phred quality score for a base call was ≤ 30 (Phred+33) and entirely excluded if the read length was ≤ 50. Two different alignments were made, one mapping the sequencing reads to the reference genome of *Mus musculus* assembly GRCm38.p6 (GCA_000001635.26), the other mapping to the reference genome of *S. aureus* strain NCTC 8325 using the spliced transcripts alignment to reference (STAR) software. This aligner features high computational speed, alignment sensitivity and precision. The minimal mapping length was set at 20 and the number of allowed mismatches was set at 6. The mapped reads aligned to the murine and the

staphylococcal reference genome were outputted in binary BAM format sorted by coordinates. Subsequently, the number of transcripts aligned to a certain gene were counted using feature counts. For the downstream analysis only uniquely mapped reads were kept, as multiple counting cannot only arise from a biological reason but also from technical artefacts.

3.2.8.4 Statistical analysis of transcriptional data

After pre-processing the raw sequencing dataset and controlling technical quality as described above, differential gene expression analysis was performed using the statistical computing software R. First, the data were normalized using a Variance Stabilizing Transformation (VST) and library size factors implemented in the R/Bioconductor DESeq2 package. The normalized dataset was used to compare the transcriptional profiles of each sample based on a Euclidean distance matrix using principal component analysis (PCA). The identification of differentially expressed genes (DEGs) for the murine and bacterial transcriptome was performed using the DESeq2 package in R. DEGs with an adjusted *p* value below 0.05 were considered statistically significant.

In Fig. 5, the workflow for pre-processing the raw data obtained from the sequencing facility is depicted in white boxes, the analysis steps performed afterwards are shown in grey boxes. The file format of the data at the different stages throughout the workflow is illustrated in the green boxes below, the tool or software that was used to perform each step is shown in the orange boxes above.

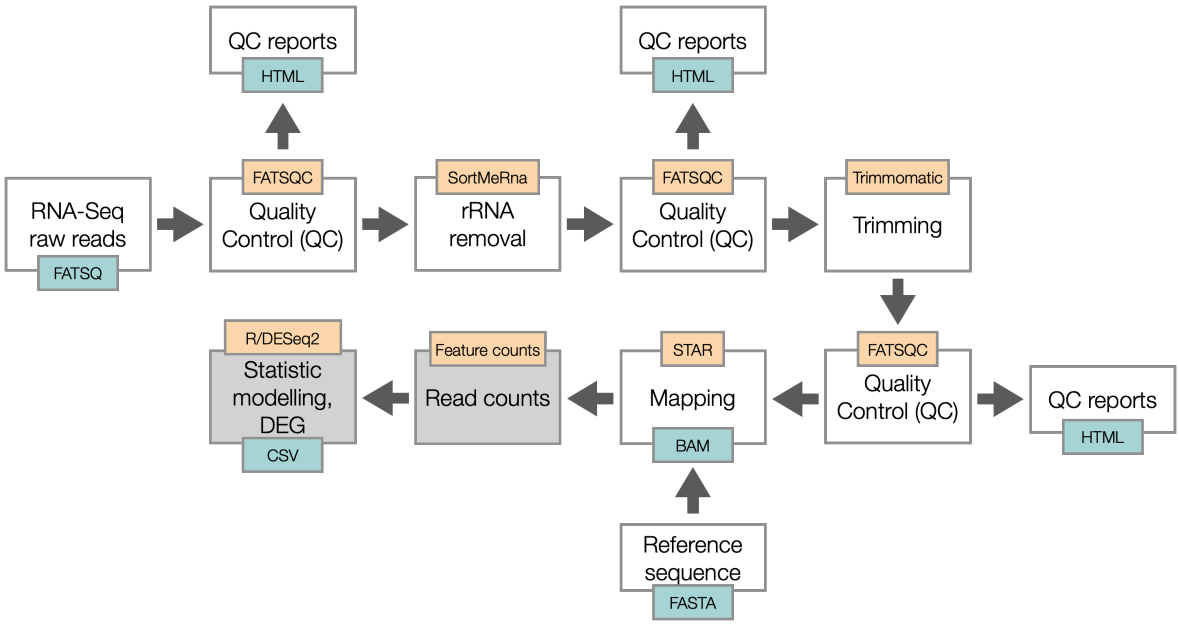

Figure 5 | RNA-Seq pre-processing and analysis workflow.
Workflow for the pre-processing (white boxes) and the analysis (grey boxes) of the RNA-Seq data of this study. Green boxes represent the data format at a given stage; orange boxes represent the tool or program that was used in each step.

3.2.9 qRT-PCR

Gene expression was quantified with a SensiFAST SYBR No-ROX one-step kit using the QuantStudio 3 real-time PCR system. Thermal cycling conditions for the PCR reaction consisted of reverse transcription at 45°C for 15 min, activation of the polymerase at 95°C for 15 min, followed by 40 cycles of denaturation (95°C, 20 sec), annealing (primer specific temperature, 20 sec), and extension (72°C, 20 sec). Primer sequences, annealing temperatures and predicted product sizes for each gene are specified in table 9. Relative gene expression levels were calculated using the comparative threshold cycle (ΔΔCT) method described by Pfaffl[341]. Values describe the change (fold) of gene expression between the treated sample and a control sample.

3.2.10 Microscopy

The sample preparation as well as the imaging using Field emission Scanning Electron Microscopy (FESEM) and Transmission Electron Microscopy (TEM) was performed in cooperation with the Central Facility for Microscopy (ZEIM) of the Helmholtz-Centre for Infection Research in Braunschweig, Germany, under the direction of Prof. Manfred Rohde. Brief description of the methods used by ZEIM staff to prepare the microscopy samples is represented below.

3.2.10.1 Preparation of colloidal gold-particles coated with BSA-protein and preloading of endosomes

100 µl BSA-protein stock solution (1 mg/ml) was added to 10 ml colloidal gold-particle solution (15 nm in size) and incubated for 30 min at RT. Cells were seeded on glass-cover slips, pre-loaded with BSA-gold complexes with a 1:16 dilution for macrophages and 1:8 dilution for epithelial cells o/n and thoroughly washed three times using cell culture medium the next day followed by infection with *S. aureus* as described in 3.2.5.

3.2.10.2 Field emission Scanning Electron Microscopy (FESEM)

After *S. aureus* infection of host cells seeded on glass cover slips, they were fixed using 2% glutaraldehyde and 3% formaldehyde in a HEPES buffer o/n at 4°C. After washing with HEPES and TE buffer, dehydration was achieved with a graded series of acetone (10, 30, 50, 70, 90 and 100%) on ice, each step for 10 min, followed by critical-point drying with liquid CO_2. Before examination in a Zeiss Merlin field emission scanning electron microscope, the samples were sputter coated with a gold/palladium film of approximately 10 nm.

3.2.1.1 Transmission Electron Microscopy (TEM)

Infected cell monolayers were fixed using a fixation solution containing 2% glutaraldehyde and 5% formaldehyde in HEPES buffer o/n at 4°C. After several washing steps with HEPES buffer, cells were fixed and contrasted with 1% aqueous osmium tetroxide for 1h at RT followed by another washing step with HEPES buffer. Cells were scrapped of the cell culture dish, pelleted and embedded in low viscosity (LV) resin according to described procedures[342]. Ultrathin sections were cut with a diamond knife, counterstained with uranyl acetate and lead citrate and examined in a Zeiss TEM910 at an acceleration voltage of 80 kV.

3.2.1.2 Confocal Laser Scanning Microscopy (CLSM)

Cells were seeded in a µ-Slide 8 well chamber and pre-loaded with Alexa Fluor® 647 labeled dextran to stain endosomal compartments o/n. The following day, cells were thoroughly washed three times using cell culture medium and infected with *S. aureus* pKikume as described in 3.2.5. For samples with photoconverted *S. aureus* pKikume, photoconversion as described in 3.2.2 was performed 30 min before microscopic analysis. Samples were examined using an inverted confocal laser scanning microscope (Zeiss SP5i). Images were obtained using the 488 argon laser line and the 561 DPSS laser line to excite the fluorescence protein mKikume green and red, respectively, whereas the 633 HeNe laser line was used to excite the endosomal tracker. In addition to the fluorescence images, differential interference contrast (DIC) images were recorded and merged with fluorescence channels to locate specific areas of interest in a specimen.

4 RESULTS

4.1 Experimental model to investigate *Staphylococcus aureus* intracellular lifestyle within macrophages

Despite not being commonly considered an intracellular pathogen, many studies have shown the capacity of *S. aureus* to survive intracellularly within host cells such as macrophages[287], neutrophils[343] and dendritic cells[344]. The capacity of *S. aureus* to survive within host cells has been broadly discussed as a potential reservoir for recurrent infections and thereby could represent a significant feature for pathogenesis. A previously described reporter system based on a photoconvertible fluorescence protein (mKikumeGR)-expressing *S. aureus* SH1000 strain (*S. aureus* pKikume) was used in the frame of this thesis to get deeper insights into the intracellular lifestyle of *S. aureus* within macrophages[326]. This system allows to link the pathogen proliferation within a host cell with the recovery of green fluorescence after photoconversion to red fluorescence and can be measured by flow cytometry and sorted by fluorescence-activated cell sorting (Fig. 6 a). *S. aureus* expressing fluorescence protein mKikumeGR can be fully photoconverted to red fluorescence by a 405nm light pulse for 90 sec and the green fluorescence efficiently recovered by actively proliferating bacteria 30 min after photoconversion (Fig. 6 b) without affecting the macrophages viability, as shown by the low level of LDH release (Fig. 6 c). Therefore, a 405 nm light pulse for 90 sec followed by a recovery time of 30 min before sampling was selected as optimal settings for photoconversion of *S. aureus* pKikume and was used for further experiments. With this reporter system, macrophage subpopulations can be identified according to the proliferation rate of intracellular bacteria by FACS analysis.

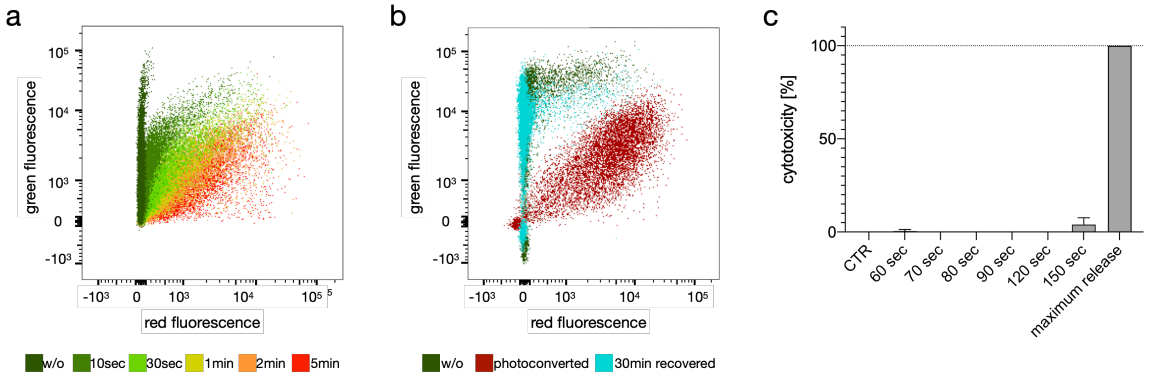

Figure 6 | Photoconversion of *S. aureus* pKikume with 405nm light pulse.
Flow cytometry plot of mKikumeGR expressing *S. aureus* showing a time course of photoconversion from green to red fluorescence using a 405nm light pulse (a). The recovery of green fluorescence after 30min recovery time is shown in (b). Effect of 405nm light pulses on the viability of immortalized macrophages determined by the release of lactate dehydrogenase (LDH) in the culture supernatant at 24h post exposure. Results are displayed as percentage of the maximum release.

In infection kinetic experiments (0h, 2h, 4h, and 24h p.I.), the proportion of *S. aureus* pKikume-infected macrophages harboring predominately red bacteria increased over time, indicating a steady decrease of the metabolic activity of intracellular bacteria (Fig. 7). At 24h p.I., the metabolic activity of the remaining intracellular bacteria was too slow to recover the expression of green fluorescence protein and exhibit a predominant red fluorescence signal. Additionally, the total number of infected macrophages was lower at 24h p.I. than at 2h and 4h p.I. The most pronounced separation between the macrophage population harboring mainly green fluorescent, metabolically active/proliferating bacteria and the macrophages mainly harboring red fluorescent metabolically inactive/non-proliferating bacteria was observed at 4h p.I. (Fig. 7).

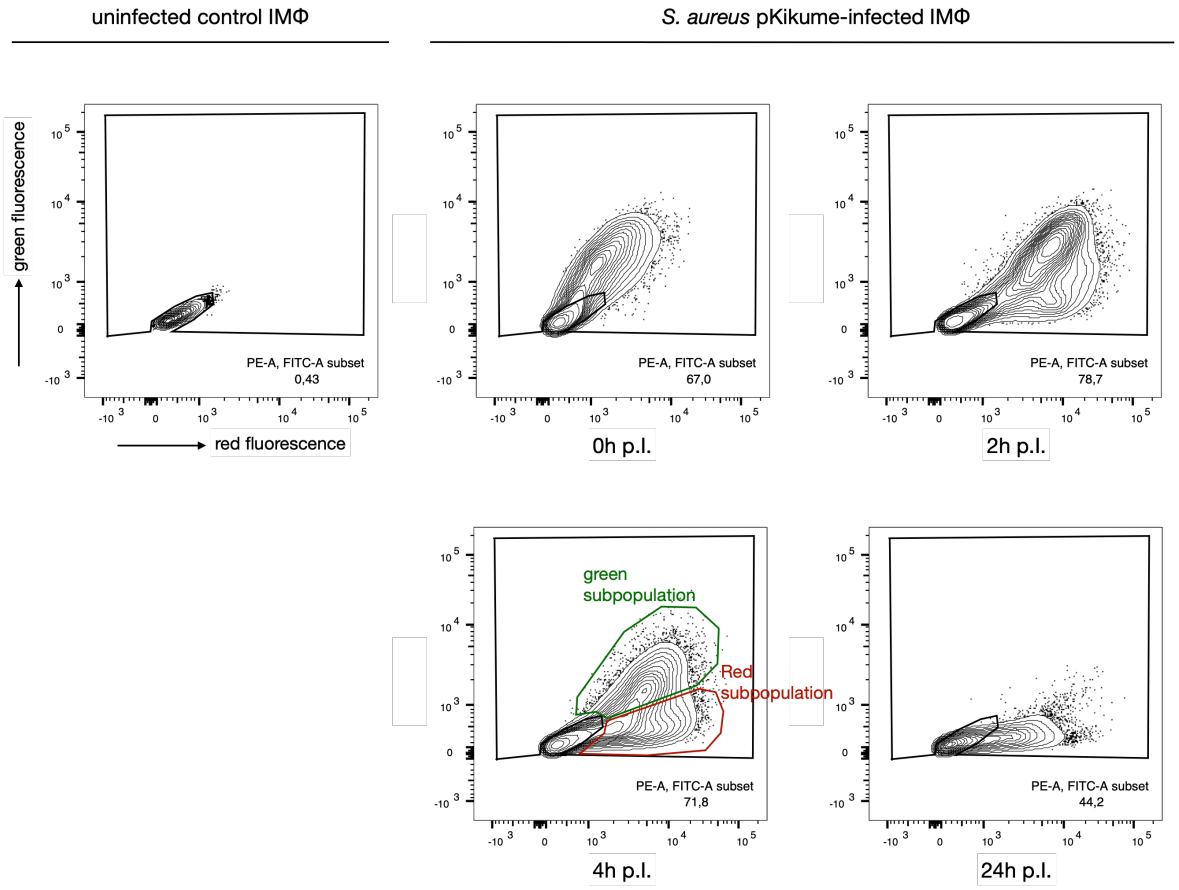

Figure 7 | Metabolic status of *S. aureus* within macrophages monitored by flow cytometry analysis over the course of infection.
Representative flow cytometry plots showing *S. aureus* pKikume-infected macrophages at increasing times p.I. The gate for infected macrophages was set based on uninfected control macrophages. At 2h p.I. the population of infected macrophages started to separate into a green subpopulation identified by the mKikume green fluorescence signal of intracellular *S. aureus* and a red subpopulation identified by the mKikume red fluorescence signal of intracellular bacteria. The separation between both subpopulations was more obvious at 4h p.I.

These two subpopulations of infected macrophages detected at 4h p.I. provided an excellent system to investigate whether the fate of intracellular *S. aureus* was predetermined by individual traits of the macrophages and/or the bacteria on a transcriptional level. For this purpose, the two infected macrophage subpopulations (termed green and red subpopulation throughout the thesis) were separated by fluorescence-activated cell sorter and subjected to dual RNA-seq analysis. A schematic overview of the experimental setup is shown in Fig. 8.

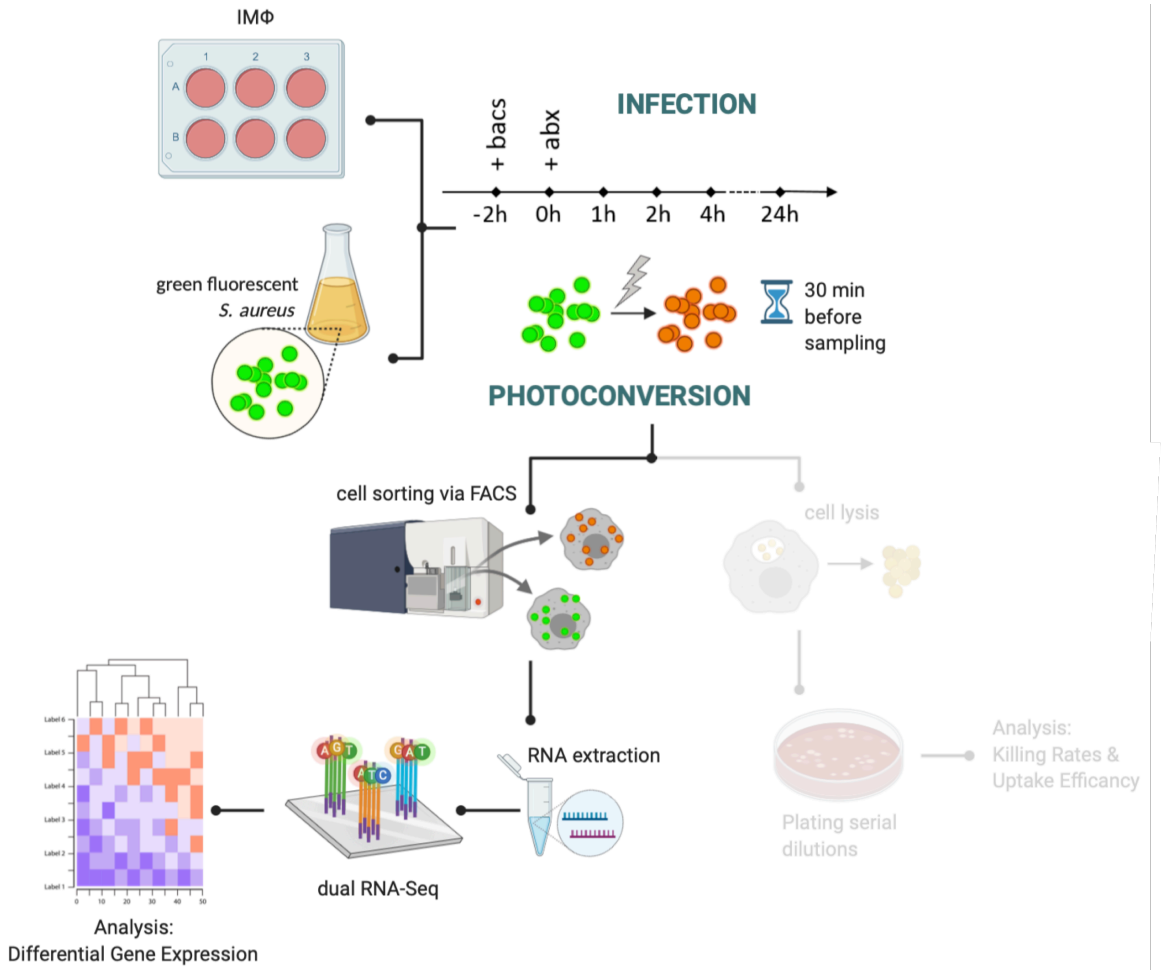

Figure 8 | Schematic outline of the experimental design for dual RNA-seq analysis.
Macrophages were infected with *S. aureus* pKikume for 2h, treated with lysostaphin/gentamicin to lyse the remaining non-internalized extracellular bacteria and subjected to a 90 sec light pulse of 405 nm wavelength to switch the bacterial fluorescence from green to red 30 min before sampling. Infected macrophages were harvested 4h after gentamicin/lysostaphin treatment and sorted via FACS into two subpopulations: the green subpopulation mainly harboring active, green fluorescent bacteria and the red subpopulation mainly harboring inactive, red fluorescent bacteria. RNA was isolated from both macrophages subpopulations and analyzed by dual RNA-seq to investigate gene expression profiles of the host and the pathogen simultaneously. This scheme was created with BioRender.com.

4.2 Dual RNA-seq analysis of infected macrophage subpopulations harboring *S. aureus* pKikume with different metabolic/proliferation states

Illumina-based dual RNA-seq methodology was used to simultaneously analyze the gene expression profiles of the host cell and the internalized *S. aureus* in the macrophage subpopulations harboring mainly red or green bacteria. The analysis of these second-generation sequencing data allows investigating the interplay between the host response to the infection and the bacterial mechanisms to survive within their host and thus the identification of factors that may be decisive for the intracellular survival of the pathogen. For this purpose, macrophages were infected with *S. aureus* pKikume at a MOI of 5:1 for 2h, harvested at 4h p.I., and sorted using a BD FACS Aria Fusion cell sorter according to the expression of green or red fluorescence of the intracellular bacteria after photoconversion. Total RNA was extracted from the green and the red macrophage subpopulations as well as from uninfected macrophages and from *S. aureus* pKikume used for the inoculum as references for the host and pathogen gene expression analysis, respectively. A minimum of 1.5 x 10^6 macrophages for each subpopulation was required for one experiment to obtain sufficient RNA that enabled the transcriptional analysis of both host and bacteria in parallel. After rRNA depletion, cDNA libraries were prepared and analyzed by Illumina NovaSeq 6000 deep-sequencing system. From the cDNA libraries, between 144.24 and 101.46 million reads per sample were obtained from which 25.22 to 58.33 million reads could be uniquely mapped to protein-coding sequences within the *Mus musculus* GRCm38.p6 (GCA_000001635.26) reference genome, and 103.71 thousand to 19.38 million reads were uniquely aligned to the *Staphylococcus aureus* NCTC 8325 transcriptome (Table 11).

Table 11 | Mapping results of samples from *S. aureus* pKikume-infected macrophage subpopulations and controls.

subpopulation	replicate	total number of reads	*M. musculus* reads	*S. aureus* reads
CTR	1	133,226,288	58,326,032	-
CTR	2	107,003,438	44,306,130	-
CTR	3	137,140,591	46,468,501	-
CTR	4	117,513,868	32,530,137	-
green	1	129,078,105	44,203,733	1,593,405
green	2	129,816,704	49,424,721	2,782,439
green	3	121,394,148	25,216,555	229,794
green	4	144,239,339	40,461,111	522,922
red	1	130,349,989	35,446,167	294,425
red	2	101,461,707	37,270,062	403,429

subpopulation	replicate	total number of reads	*M. musculus* reads	*S. aureus* reads
red	3	140,321,397	42,046,848	103,711
red	4	125,287,483	39,911,330	365,333
inoculum	1	40,035,465	-	12,193,921
inoculum	2	48,794,896	-	19,378,965
inoculum	3	42,166,079	-	13,602,861
inoculum	4	47,006,744	-	13,984,955

A graphic illustration of the mapping results is depicted in Figure 9, with the reads aligned to the host reference genome in Fig. 9 a and the reads aligned to the pathogen reference genome in Fig. 9 b.

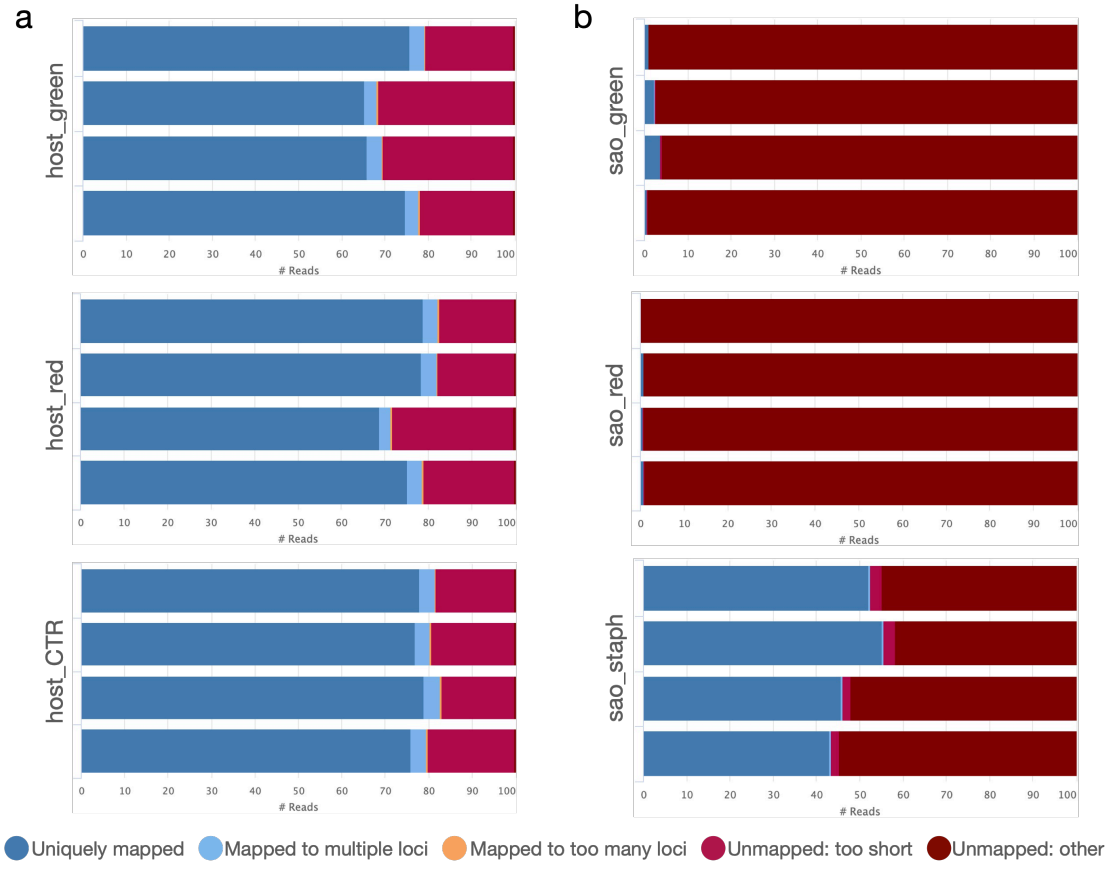

Figure 9 | Alignment Score of *S. aureus* pKikume-infected macrophage subpopulations and control samples displayed in percentage.
Sequencing data quality assessment showing the percentage of reads within each sample group (green, red and control/staph) aligned to the host (*Mus musculus*) reference genome (a) and the pathogen (*Staphylococcus aureus*) reference genome (b).

4.3 Transcriptional response of infected macrophage subpopulations harboring *S. aureus* pKikume with different metabolic/proliferation status

4.3.1 Biological quality assessment of the host transcriptional datasets

Principal component analysis (PCA) was performed to assess whether transcriptomic datasets and their differences are related to biological means or the experimental design and no interfering confounding factors, e.g. sampling date, or artefacts bias the result[345,346]. The PCA plots visualize the relationship between transcriptional datasets of macrophage subpopulations harboring mainly green or mainly red *S. aureus* as well as those of uninfected control macrophages. The PCA clustering analysis reveals a clear separation between infected macrophages harboring predominately red (Fig. 10 a) or green (Fig. 10 b) *S. aureus* and uninfected cells along PC1. This underscores the impact of *S. aureus* infection in the transcriptional response of macrophages, independently of the metabolic state of the bacteria that they harbor (red or green). On the other hand, PCA clustering comparing infected macrophages harboring mainly red with those harboring mainly green bacteria shows an intermixing of samples from both groups (Fig. 10 c), suggesting an overlapping in the gene expression profiles of these samples. Based on this analysis, there seem to be no heterogeneity within the host cell population that may be responsible for the differences in their ability to control phagocytosed *S. aureus*.

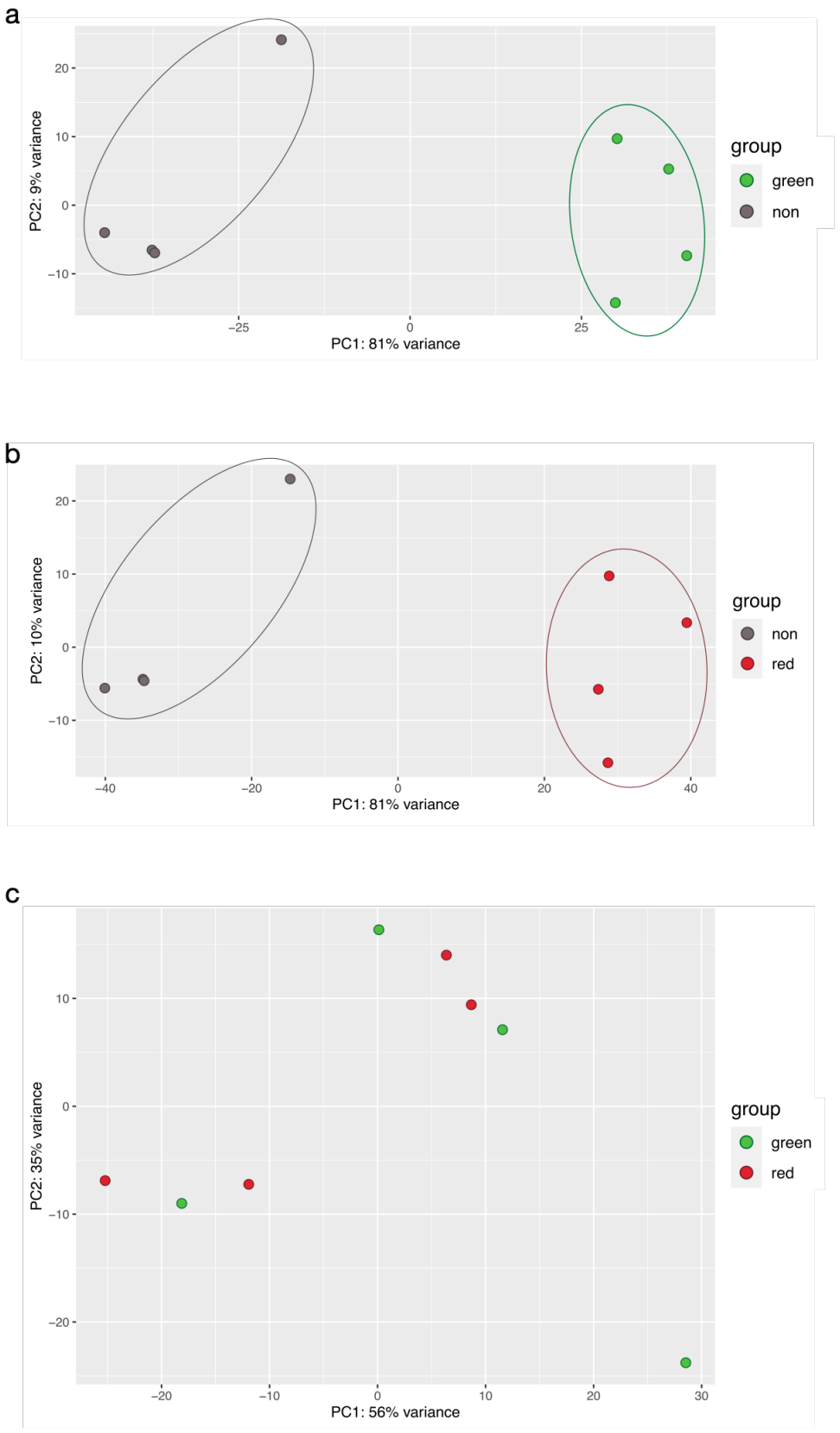

Figure 10 | Principal component analysis of host transcriptomes from *S. aureus* infected green or red macrophage subpopulation and uninfected controls.
PCA clustering of the transcriptomes of macrophage subpopulations harboring mainly green or red *S. aureus* compared to the transcriptome of uninfected control macrophages (non) (a, b) or compared to each other (c) based on a Euclidean distance matrix of normalized RNA-seq data. Circles enclosing replicates that cluster together. Each dot represents one biological replicate.

A heatmap visualizing the expression of genes across the samples is shown in Fig. 11, supporting the substantial differences between infected macrophages (green or red subpopulation) and the uninfected control samples. However, no significant differences between macrophages harboring mainly red or mainly green bacteria was noticable.

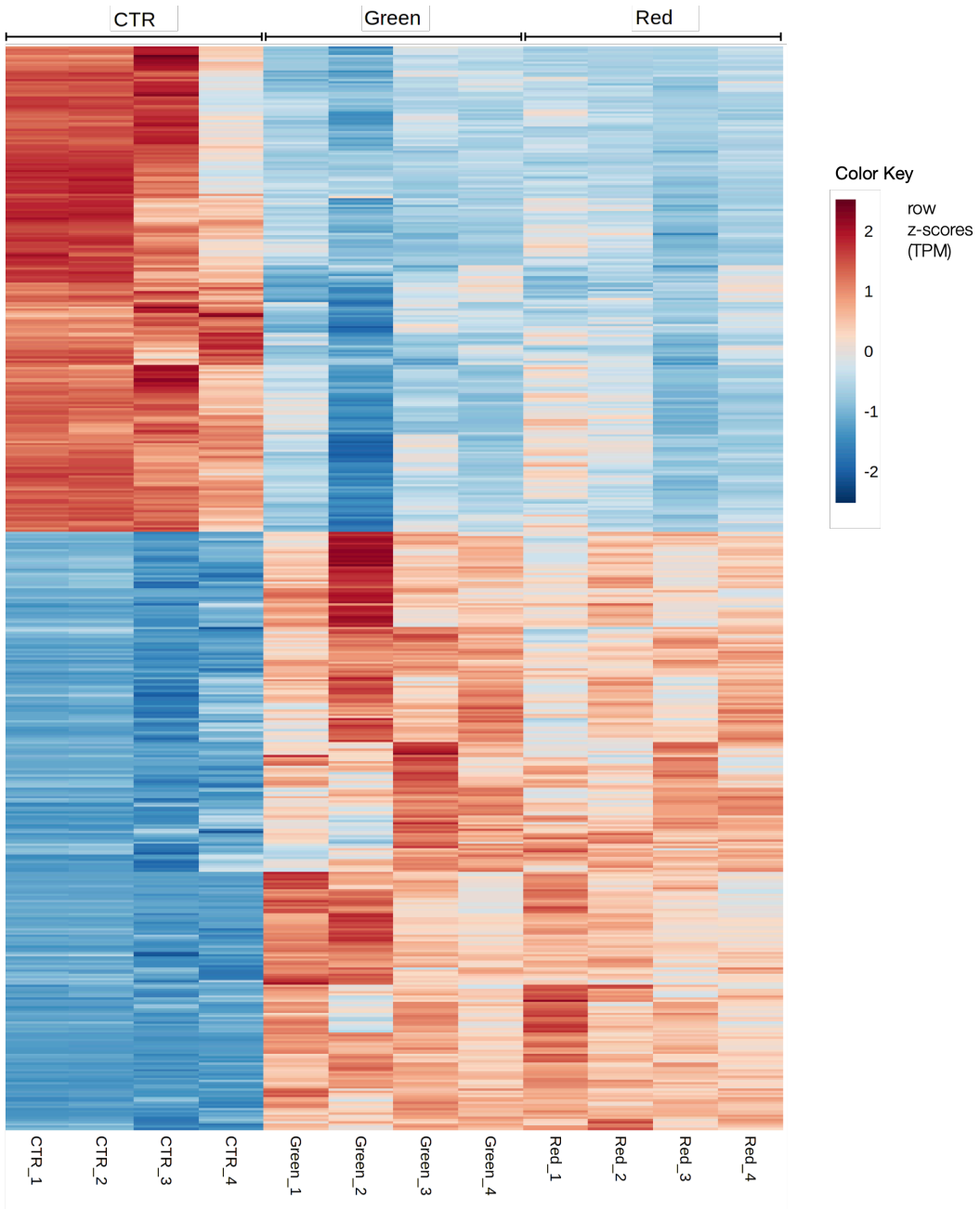

Figure 11 | Heatmap of the RNA-seq global host gene expression pattern in macrophage subpopulations harboring mainly green or red *S. aureus* and in uninfected control macrophages.
Heatmap showing differential expression of the top 500 genes between macrophage subpopulations harboring mainly green (Green) or red (Red) *S. aureus* and uninfected control macrophages (CTR). Color key represents z-score normalized transcripts per million (TPM).

4.3.2 Differential gene expression analysis of the different *S. aureus* pKikume-infected macrophage subpopulations

Differential gene expression analysis was performed to closely dissect the macrophage response to *S. aureus* infection in both red and green subpopulations. For the identification of genes that are differentially expressed (DEGs) between infected subpopulations of macrophages and their uninfected mock-treated controls (CTR), the DESeq2 algorithm[347] was used. *S. aureus* infection induced significant changes in gene expression in both macrophage subpopulations. Genes were considered up-regulated at $\log_2$(fold change) > 2, BaseMean > 10 and an adjusted *p*-value < 0.05 and down-regulated at $\log_2$(fold change) < -2, BaseMean > 10 and an adjusted *p*-value < 0.05 (Fig. 12). A total of 522 genes were found up-regulated and 499 down-regulated in respect to uninfected controls in macrophages containing green bacteria (supplement, Table S1) and 429 up-regulated genes and 405 down-regulated in respect to uninfected controls in macrophages containing red bacteria (supplement, Table S2). No differentially expressed genes were found when comparing infected macrophages harboring mainly red with those harboring mainly green bacteria. This confirmed the results of the PCA analysis and highlights the fact that macrophages harboring metabolically active *S. aureus* pKikume (green) did not differ at the transcriptional level from those harboring metabolically inactive or dead bacteria (red).

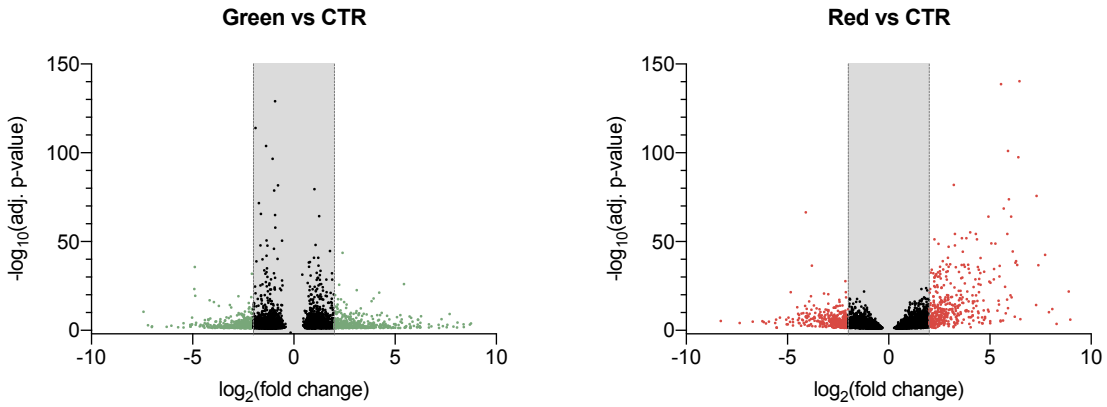

Figure 12 | Volcano plots of the host differential gene expression analysis between each macrophage subpopulation and the uninfected controls.
Volcano plot of changes in gene expression between the green macrophage subpopulation (left) or the red macrophage subpopulation (right) in respect to uninfected control samples. The $\log_2$ fold change of each gene is plotted against negative $\log_{10}$ *p*-value of differential gene expression. Each gene is represented by a dot. Genes identified as DEGs by DESeq2 analysis with a Benjamini Hochberg adjusted *p*-value is < 0.05 and $\log_2$ fold change >2 or <-2 are labeled in green (left) or red (right).

To obtain more information on the functional organization of the genes that were up- or down-regulated in response to *S. aureus* infection in both macrophage subpopulations, they

were subjected to functional enrichment analysis. This analysis was based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation[348] and permits mapping the identified DEGs to pathway maps of molecular interactions, reactions, and relations for various organisms. Functional analysis of the upregulated genes showed enrichment in biological processes associated with the inflammatory response such as cytokine-cytokine receptor interaction, TNF signaling pathway, Toll-like receptor signaling pathway, chemokine signaling pathways, among others (Table 12). Furthermore, pathways associated with the response to several infections, such as Influenza A, Malaria, Tuberculosis, etc., were also found to be enriched in both macrophage subpopulations (Table 12). A selection of up-regulated genes encoding factors involved in these pathways is shown in Fig. 13.

Table 12 | KEGG pathway annotation of upregulated DEGs between infected green or red macrophage subpopulation and uninfected control macrophages.

KEGG pathway	green subpopulation		red subpopulation	
	adjusted p-value	number of DEGs	adjusted p-value	number of DEGs
Cytokine-cytokine receptor interaction	1,90E-15	33	8,00E-15	30
TNF signaling pathway	2,00E-14	23	8,00E-15	22
Influenza A	1,90E-09	22	2,90E-08	19
Herpes simplex virus 1 infection	5,60E-08	22	2,90E-09	22
Toll-like receptor signaling pathway	5,90E-08	16	5,50E-08	15
Malaria	9,80E-07	11	2,80E-06	10
Chagas disease	3,90E-06	14	4,30E-06	13
Chemokine signaling pathway	1,10E-05	18	5,10E-06	17
Leishmaniasis	1,10E-05	11	2,10E-04	9
Measles	1,20E-05	15	1,20E-03	11
Tuberculosis	4,70E-05	16	n.s.	n.s.
African trypanosomiasis	8,00E-05	8	n.s.	n.s.
Rheumatoid arthritis	8,10E-05	11	9,20E-04	9
NOD-like receptor signaling pathway	2,30E-04	9	6,30E-04	8
Inflammatory bowel disease	2,80E-04	9	n.s.	n.s.
NF-kappa B signaling pathway	2,90E-04	11	5,70E-04	10
Salmonella infection	2,90E-04	10	3,60E-03	8
Jak-STAT signaling pathway	3,90E-04	13	1,30E-04	13
Cytosolic DNA-sensing pathway	4,00E-04	9	1,20E-03	8
Transcriptional misregulation in cancer	1,20E-03	13	4,00E-04	13
Legionellosis	1,20E-03	8	n.s.	n.s.
Toxoplasmosis	2,30E-03	10	4,00E-03	9

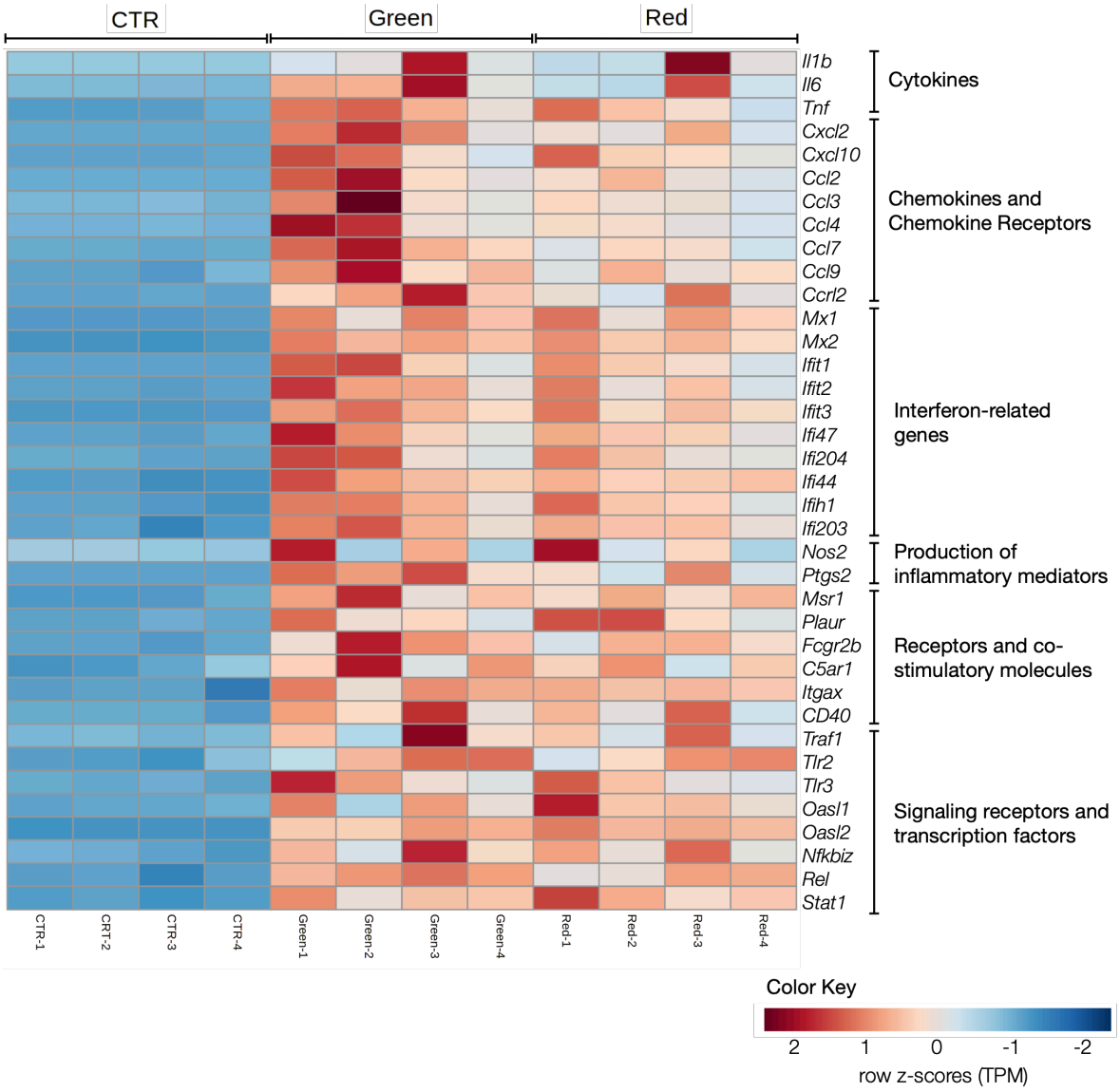

Figure 13 | Heatmap of selected up-regulated genes in infected macrophages (green and red) versus uninfected controls involved in enriched KEGG pathways associated to inflammation.
Heatmap illustrating the gene expression of selected up-regulated genes involved in KEGG pathways that are found to be enriched in the green and the red macrophage subpopulation and are associated with an inflammatory response. Color key represents z-score normalized TPM.

The gene expression profile of the macrophage subpopulations recapitulated many features of the well-described innate immune response to *S. aureus.* In general, innate immune responses are triggered upon recognition of conserved pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), including toll-like receptors (TLRs). Among the different TLRs, TLR2 has been shown to play an essential role in sensing cell wall structures of *S. aureus* lipoteichoic acid (LTA) and peptidoglycan[205]. Signaling via surface-exposed TLR2 triggers the activation of downstream signaling cascades leading to the activation of transcription factor NF-κB, which controls the induction of proinflammatory

cytokines and chemokines as well as the upregulation of co-stimulatory molecules and other inflammatory mediators[349]. TLR2 signaling seems to be an important pathway in the recognition of *S. aureus* by both macrophage subpopulations as shown by the up-regulation of the gene encoding TLR2 (*Tlr2*), several genes encoding cytokines (*e.g. Il6, Il1b, Tnf*) and chemokines (*e.g. Cxcl2, Cxcl10, Ccl2, Ccl3, Ccl4, Ccl7, Ccl9).* In addition to surface signaling, TLR2 can also signal from endosome to activate interferon regulatory factors (IRF) that lead to the activation of type I Interferon[202]. The up-regulation of a large number of interferon-related genes indicate TLR2 endosomal signaling was also induced in both macrophage subpopulations. Interestingly, the gene encoding the TLR3 receptor (*Tlr3)* was also found significantly up-regulated in macrophages upon infection of *S. aureus.* Whereas TLR2 recognizes bacterial cell wall components, TLR3 has been shown to recognize bacterial double-stranded RNA in endosomes after phagocytosis[350] and can also contribute to the induction of interferon-related genes[198].

No KEGG pathway enrichment patterns were observed in down-regulated genes neither in macrophages containing mostly green nor in those containing mostly red bacteria.

Since no differences were observed in the transcriptional response between macrophages harboring metabolically active green *S. aureus* and those harboring metabolically inactive or dead *S. aureus*, the possibility that some macrophages had better capacity to kill the phagocytosed bacteria while others were permissive to intracellular bacterial survival could not be proven in this study. Instead, it can be speculated that the infecting bacterial population contains subpopulations of bacteria that vary in their capacities to survive and proliferate intracellularly within macrophages.

4.4 Transcriptional response of intracellular metabolically active, green and metabolically inactive, red *S. aureus* pKikume during infection of macrophages

4.4.1 Biological quality assessment of the pathogen transcriptional datasets

In a similar way as for the host transcriptome, PCA was performed to determine if there are biases in the RNA-seq dataset that could affect the results and if there are outliers within the sample groups. PCA plots of the transcriptomes of green (Fig. 14 a) or red (Fig. 14 b) intracellular *S. aureus* vs. the bacterial inoculum showed that samples in each subpopulation clustered tightly together along PC1, which indicates consistency in gene expression between the replicates except for one outlier in each group. Furthermore, PC1, which explains the main axis of variance between samples, clearly separated intracellular (green or red) *S. aureus* and the bacterial control samples, indicating that the gene expression profile of intracellular *S. aureus* (red or green subpopulation) differed significantly from that of the bacterial inoculum and that the milieu (intra- or extracellular) is the key variable in this data.

PCA was also performed on the RNA-Seq data of intracellular metabolically active, green *S. aureus* compared to that of intracellular metabolically inactive, red bacteria. The PCA plot depicted in Fig. 14 c identified clustering of the replicates more along PC2, with one outlier along the PC1 axis of the red population and one outlier along PC2 in the green group. This indicates a difference in the gene expression profiles between intracellular red and green *S. aureus* that is not due to their intra-/extracellular milieu.

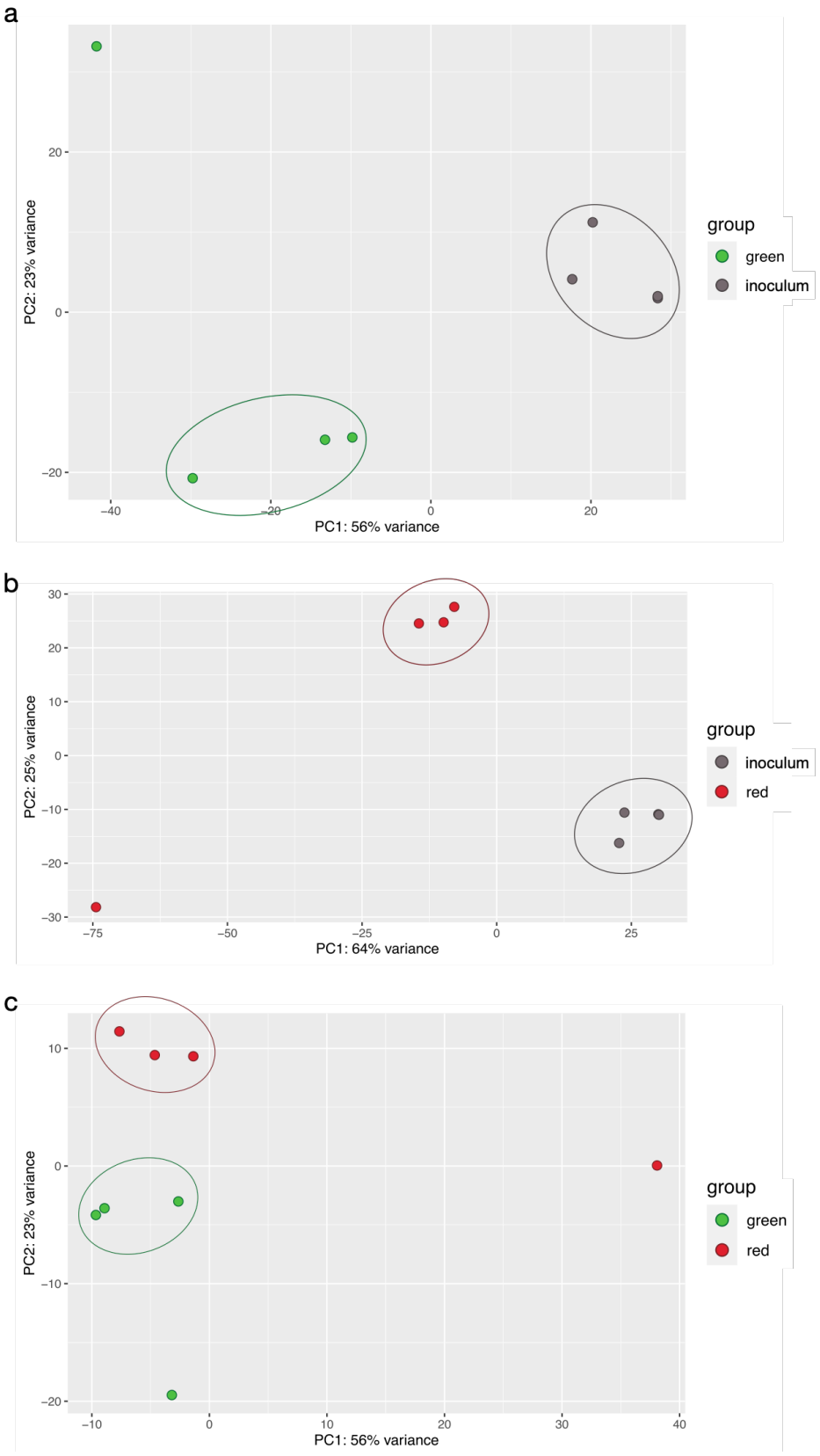

Figure 14 | Principal component analysis of transcriptomes from intracellular green or red *S. aureus* and inoculum control bacteria.
PCA clustering of the transcriptomes of intracellular green or red *S. aureus* compared to the transcriptome of *S. aureus* in the inoculum (a, b) or compared to each other (c) based on a Euclidean distance matrix of normalized RNA-seq data. Circles enclosing replicates that cluster together. Each dot represents one biological replicate.

4.4.2 Analysis of differentially expressed genes between intracellular metabolically active (green) or intracellular metabolically inactive (red) *S. aureus* pKikume and control inoculum bacteria

As for host transcriptomic profiles, differential gene expression analysis was also performed for investigating the differences between intracellular mainly green or red *S. aureus* subpopulations and the bacteria from the original inoculum. Here, a total of 563 DEGs was identified as significant (log_2 fold change > 1 or < -1, adjusted *p*-value < 0.05) for the green subpopulation and 735 DEGs for the red subpopulation. Both groups shared a set of 423 genes with significant changes in response to the intracellular milieu, 140 DEGs are uniquely for the green and 312 for the red subpopulation (Fig. 15, supplement Table S3 and S4).

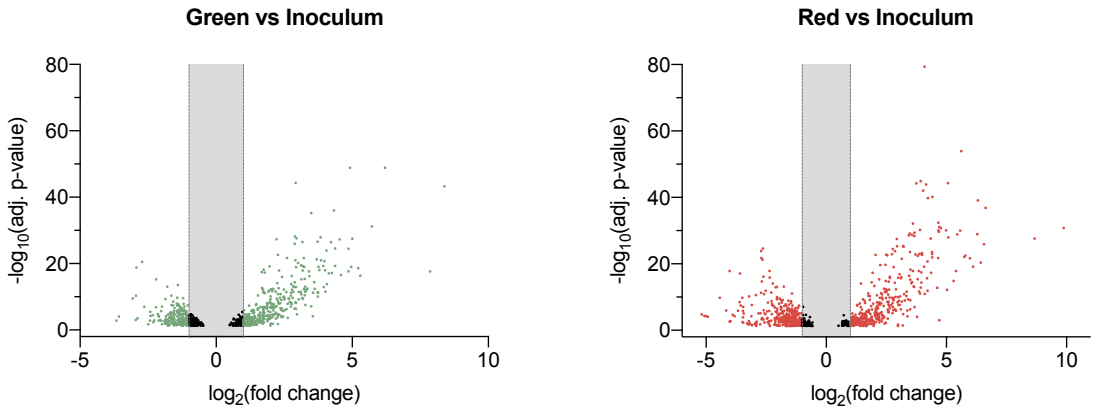

Figure 15 | Volcano plots showing the differential expression of genes between intracellular green or red *S. aureus* and *S. aureus* from the inoculum.
Volcano plot of changes in gene expression between the intracellular green (left) or the red (right) *S. aureus* and *S. aureus* from the inoculum. The log2 fold change of each gene is plotted against the negative log_{10} adjusted *p*-value of differential gene expression. Each gene is represented with a dot which is colored in green or red when the Benjamini Hochberg adjusted *p*-value < 0.05 and the log_2 fold change is > 1 or < -1.

To survive within macrophages, *S. aureus* needs to express an array of virulence factors that enable the bacterium to avoid destruction by the antimicrobial mechanisms of the phagocytic cell. The set of genes differentially expressed between intracellular *S. aureus* and inoculum bacteria and which exhibited similar expression levels in red and green bacteria was used to identify the common intracellular response/survival pathways. A total of 267 genes were found up-regulated (Log_2 fold change > 1, adjusted *p*-value < 0.05) and 155 down-regulated (Log_2 fold change < -1, adjusted *p*-value < 0.05) by both red and green intracellular *S. aureus* in respect to the bacteria of the inoculum. One of this common DEGs was found up-regulated in the green and down-regulated in the red intracellular *S. aureus* compared to the inoculum (supplement, Table S3 and S4). Many of the up-regulated genes were related to

biosynthesis of amino acids, oligopeptides and sugar transport systems, molecular chaperones involved if the refolding or degradation of damaged proteins, virulence factors, regulatory systems and central metabolism (Fig. 16, appendix Table A1). Among the genes related to amino acids biosynthesis were those encoding factors involved in the synthesis of methionine, lysine and threonine (*lysC, asd, dapABD*) as well as those of the *ilv-leu* operon (*ilvA2BCD, leuBCD*) encoding proteins involved in the biosynthesis of branched-chain amino acids (BCAA; isoleucine, leucine, valine) (appendix Table A1). This indicates that the levels of these amino acids in the intracellular environment may not be sufficient to supply the physiological requirements of *S. aureus* and the bacterium needs to synthesize them. Intracellular *S. aureus* can also obtain amino acids from host oligopeptides that can be imported via transporter systems. Two genes of the Opp-3 operon (*opp-3A, opp3F*), which is able to import oligopeptides comprising 3 to 0 amino acids[351] were found up-regulated in intracellular *S. aureus* (appendix Table A1). The capacity to extract carbon sources from the host is critical for *S. aureus* intracellular survival. For this purpose, intracellular *S. aureus* up-regulates several transport systems for scavenging gluconate (*gntP*), sucrose (*scrA*), glucose (*glcB*) and maltose (*malF*) from the host (appendix Table A1). The capacity of *S. aureus* to survive intracellularly is also dependent on its capacity to repair the proteins damaged by the antimicrobial mechanisms of macrophages. This is achieved by molecular chaperone such as DnaK, which plays an important role in protein folding and refolding of damaged proteins in cooperation with co-chaperones DnaJ and GrpE[352] and GroEL/GroES[353] .The genes encoding these chaperones and co-chaperons as well as the gene encoding their regulator HrcA (*hrcA*) were found up-regulated in intracellular *S. aureus* (appendix Table A1). In addition, several genes coding for members of the stress response-related Clp ATPases (*clpB, clpC, clpL*) were found (appendix Table A1).

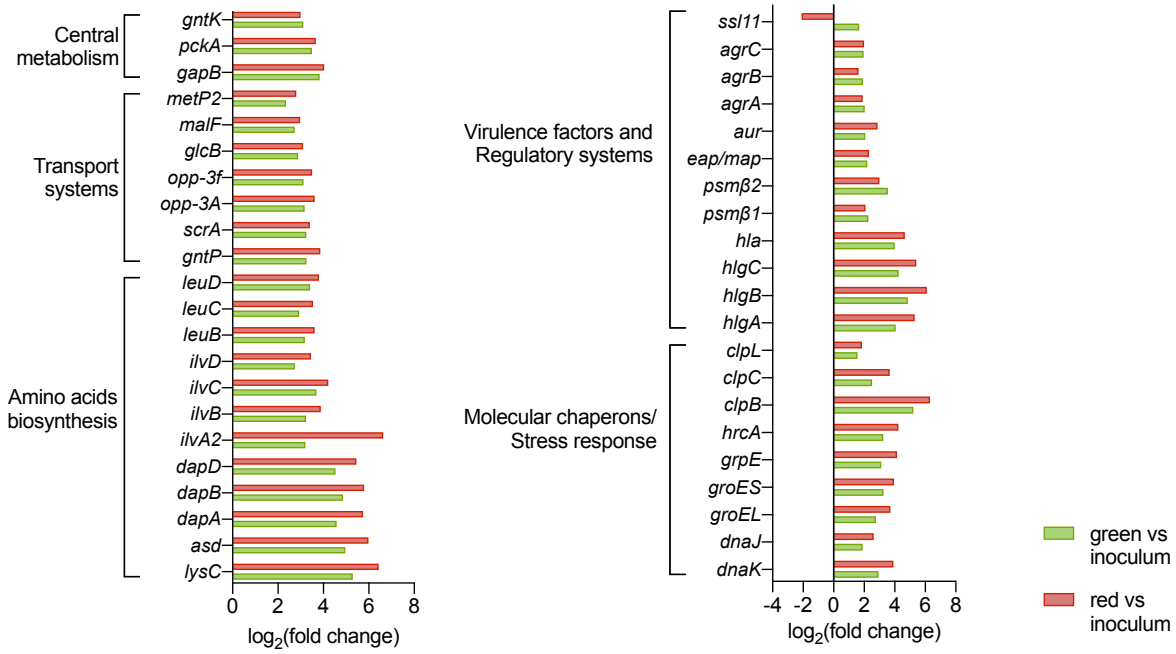

Figure 16 | Selected DEGs between intracellular *S. aureus* (either green or red subpopulation) and *S. aureus* from the inoculum.
Expression levels of a set of DEGs between intracellular *S. aureus* of the green (green bars) or the red (red bars) *S. aureus* subpopulations and the *S. aureus* from the inoculum shown as $\log_2$ fold change.

Regarding virulence factors, the genes encoding cytolysins such as *α*-hemolysin (*hla*), ɣ-hemolysin subunits (*hlgA, hlgB, hlgC*) and two phenol-soluble modulins (PSM) beta (*psmβ1, psmβ2*) were up-regulated in intracellular *S. aureus* (appendix Table YY). Interestingly, the expression of *α*-hemolysin has been shown to be important for *S. aureus* evasion of phagosomes and resistance against the killing mechanisms of macrophages[287], although this issue remains highly controversial[289,354]. The ɣ-hemolysin locus consists of three genes (*hlgA, hlgB, hlgC*) and can display two bi-components combinations to produce the leukocidins HlgAB and HlgCB[116]. The ɣ-hemolysin genes have been shown to be induced in *S. aureus* upon neutrophil phagocytosis[225]. PSMs are encoded in two operons, PSM*α* and PSM*β*, with PSMα operon comprising four open reading frames (*psmα1-4*) and PSMβ comprising two open reading frames (*psmβ1* and *psmβ*2)[137]. Only the genes encoding PSMβ were up-regulated by *S. aureus* within macrophages. This is of interest because the expression of PSMβ has been shown to be involved in *S. aureus* intracellular survival[289].

The expression of *hla,* ɣ-hemolysin subunits and PSM is largely controlled by the accessory gene regulator (Agr), the most prominent regulatory system of *S. aureus*[355]. Expression of Agr has been shown to be critical for the survival of *S. aureus* within macrophages[287]. The Agr genetic locus comprises an operon with two divergent transcriptional

units (RNAII and RNA III) driven by two separate promoters, P2 and P3, respectively[149]. Only the genes within the RNAII operon were up-regulated by intracellular *S. aureus.*

Other genes up-regulated by intracellular *S. aureus* were those coding for the metalloprotease aureolysin (*aur*) as well as the gene encoding the extracellular adherence protein Eap (*eap/map*). Whereas expression of aureolysin has been shown to be important for *S. aureus* to survive within macrophages[287], the potential role of Eap remains to be determined.

The only gene found to be up-regulated in green and down-regulated in red intracellular *S. aureus* compared to inoculum bacteria encodes for the superantigen like protein 11 (*ssl11*). So far, SSL11 was found to play a role in immune evasion as several studies have reported the capacity of SSL11 to influence neutrophil function in multiple ways including inhibition of neutrophil activation and rolling[356–358], but an intracellular function of this factor remains undetermined.

4.4.3 Analysis of differentially expressed genes between intracellular metabolically active (green) and intracellular metabolically inactive (red) *S. aureus*

To determine differences in the transcriptional profiles of intracellular *S. aureus* with different metabolic states, DEGs between intracellular metabolically active green *S. aureus* and intracellular metabolically inactive red *S. aureus* were identified using the DESeq2 algorithm. A total of 171 genes were found differentially expressed between intracellular green and red bacterial subpopulations (adjusted p-value < 0.05), of which 124 exhibited higher expression in the green than in the red subpopulation and 47 exhibited higher expression in the red than in the green subpopulation (Fig. 17, supplement Table S5).

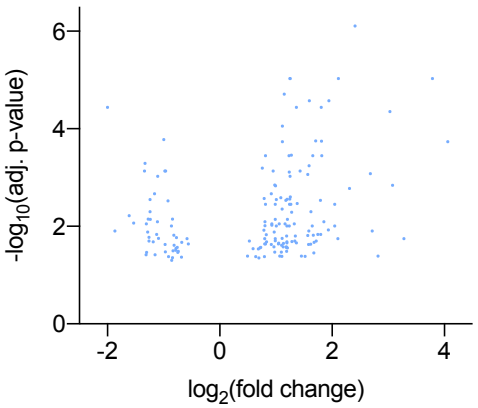

Figure 17 | Volcano plot showing the differential expression of genes between intracellular green and red *S. aureus* subpopulations.
Volcano plot of changes in gene expression between the intracellular green (left) and the red (right) *S. aureus*. The log2 fold change of each gene is plotted against the negative $\log_{10}$ adjusted p-value of differential gene expression. Each gene with a Benjamini Hochberg adjusted p-value < 0.05 is represented with a dot.

Among the genes with higher expression in green bacteria are those involved in protein synthesis (*e.g. trnaG, trnaL, trnaH, trnaS, trnaD,*), cell division (*e.g. ftsL, ftsW, ftsK, ftsA, sle1, divlB, spoIIIE*, *mraZ*), DNA replication (*e.g. holB, cshA, yabA, topB*) and cell wall synthesis (*lrgA, mraY, pbp1, murD*) (Fig. 18, appendix Table A2). This indicates that green *S. aureus* are more actively synthetizing proteins and undergoing cell division than red *S. aureus*. Among the genes with higher expression in red *S. aureus* were those involved in stress response such as oxidative stress (e.g. *kat, sodA, ahpC*) and misfolded protein degradation (*e.g. ctsR, mcsB and clpC*) (Fig. 18, appendix Table A2). Because these genes are induced in response to environmental stresses[359], it can be anticipated that the red *S. aureus* encounter more harsh conditions in the intracellular milieu than green *S. aureus* such as high levels of reactive oxygen species that can cause damage on proteins and cell wall. Consequently, the bacteria need to induce damage repair mechanisms as well as to produce catalase (encoded by *kat*), superoxide dismutase (encoded by *sod2*) and alkyl hydroperoxide (encoded by *ahpC*) to neutralize them.

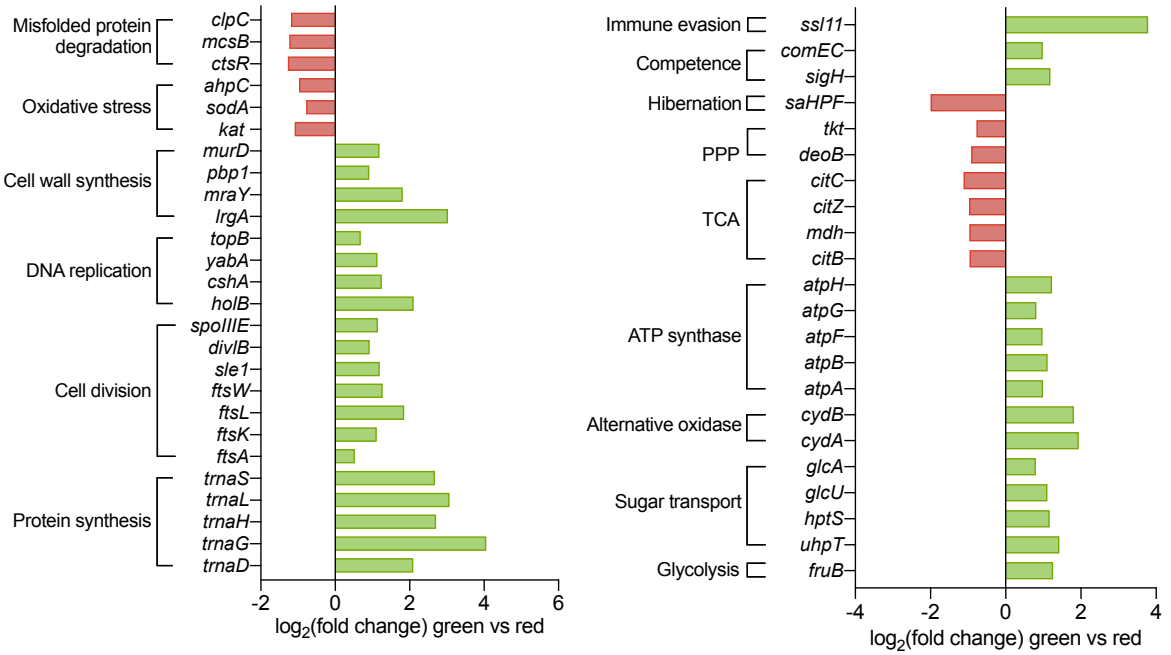

Figure 18 | Selected DEGs between intracellular green and red *S. aureus* subpopulations.
Expression levels of a set of DEGs between intracellular green and red *S. aureus* subpopulations shown as $\log_2$ fold change. Positive values, depicted as green bars, indicate higher expression levels in the green subpopulation; negative values, depicted as red bars, indicate higher expression levels in the red subpopulation.

The differential expression of genes related to metabolism indicated that the nutrient availability differed between the intracellular environment where red and green *S. aureus* are located. Thus, green *S. aureus* exhibited higher expression of genes encoding enzymes involved in glycolysis (*fruB*) and sugar transport (*uhpT, glcU, glcA*) (Fig. 18, appendix Table A2). This

suggests that green *S. aureus* is located in a nutrient-rich environment. Furthermore, the higher expression of genes encoding members of the alternative oxidase (*cydAB*) and ATP synthase (*atpABFGH*) supports the notion of a microaerobic environment (Fig. 18, appendix Table A2)[360]. In contrast, the higher expression of genes related to tricarboxylic acid (TCA) cycle (*mdh, citBCZ*) and pentose phosphate pathway (*deoB, tkt*) in red bacteria (Fig. 18, appendix Table A2) indicates that they may be located in a nutrient-poor intracellular compartment, where tricarboxylic acid (TCA) cycle activity is required for the catabolism of nonpreferred carbon sources[361]. The higher expression of the gene encoding a hibernation promoting factor SaHPF (*saHPF*) in red compared with green *S. aureus* is a further indicator of harsher intracellular environment encounter by the red bacteria (Fig. 18, appendix Table A2). During extreme stress conditions and nutrient deprivation, the 70S ribosomes are converted into inactive 100S ribosome dimers by associating with SaHPF, which helps the pathogen to reduce energy[362].

Interestingly, the genes encoding the alternative sigma factor H (*sigH*) and the genetic competence genes *comEC* were expressed to a higher extent by green than by red *S. aureus* (Fig. 18, appendix Table A2). Expression of *sigH* seems to be induced by the *in vivo* environment since its expression is not detectable under standard laboratory culture conditions[167]. SigH is required for the induction of competence genes in *S. aureus*[168] and bacteria expressing SigH become competent for transformation by foreign DNA[167]. This is interesting because competence is thought to be important for cell survival as the imported DNA can serve as nutrient as well as for providing beneficial genes.

As already mentioned in the previous section, the gene encoding SSL11 was induced in green but not in red intracellular *S. aureus* when compared with the bacteria from the inoculum. Consequently, the expression of this gene is significantly greater in green than in red intracellular bacteria. However, the specific function of SSL11 in the intracellular milieu remains to be established.

4.4.4 Validation of RNA-seq data by qRT-PCR

Quantitative real-time PCR was performed using a small set of selected genes to validate the gene expression data obtained by RNA-seq of intracellular green and red *S. aureus* compared to control bacteria from the inoculum. The results of the qRT-PCR are depicted in Fig. 19 and confirm the results of the RNA-seq analysis.

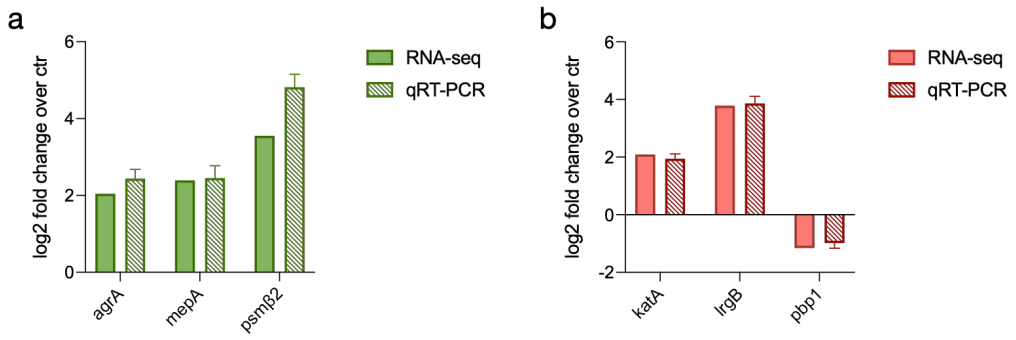

Figure 19 | Validation of changes in gene expression of selected genes by qRT-PCR.
Staphylococcal RNA-seq data were validated by comparing the fold changes [log_2] in expression of selected genes in intracellular green (a) or the red (b) *S. aureus* subpopulations versus uninfected control samples identified by DESeq2 analysis of RNA-seq data (filled bars) with the fold changes [log_2] of the same genes calculated by qRT-PCR (patterned bars). The filled bares represent the mean value of four independent experiments. The patterned bars for the green population (a) represent the mean of four independent experiments, the patterned bars for the red population the mean value of four independent experiments with error bars displaying the SD.

4.5 Intracellular survival of *S. aureus* pKikume within macrophages is influenced by the specific internalization pathway engaged

The results of the dual RNA-seq analysis indicate that the fate of intracellular *S. aureus* was not determined by variability within the macrophage population. Furthermore, they suggest that the differences in the transcriptional response between intracellular metabolically inactive red and metabolically active/proliferating green *S. aureus* most probably resulted from the different environmental stress conditions that they encountered within the subcellular compartment where they are located. To further investigate the intracellular lifestyle of *S. aureus* pKikume within professional phagocytes like macrophages the same experimental infection model as for the RNA-Seq was used to determine intracellular bacterial viability in plating experiments as displayed in Fig. 20.

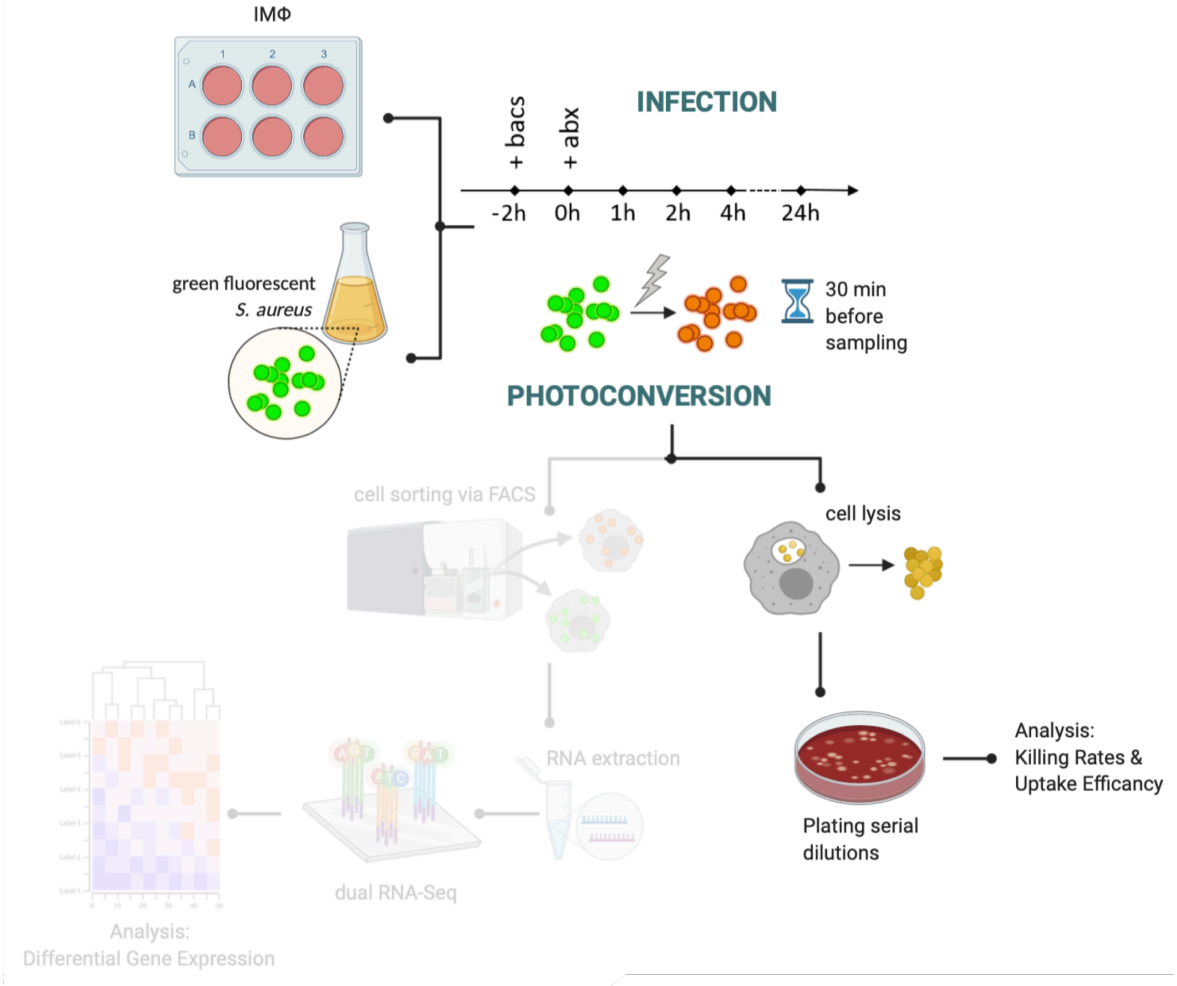

Figure 20 | Schematic outline of the experimental design for determination of intracellular *S. aureus* viability. Macrophages were infected with green fluorescent *S. aureus* SH1000 pKikume for 2h, lysostaphin/gentamicin treated to lyse all extracellularly remaining bacteria and photoconverted by a 90sec light pulse of 405 nm wavelength to switch the bacterial fluorescence to red 30 min before sampling. At specific times after infection, infected macrophages were lysed and serial dilutions were plated on sheep blood agar to determine bacterial CFU. This scheme was created with BioRender.com.

The main mechanism used by macrophages to uptake and kill microbial pathogens is through phagocytosis[228]. However, the flow cytometry analysis depicted in Fig. 7 indicates that a subpopulation of *S. aureus* bacteria had the capacity to circumvent the phagocytic pathway of macrophages and gain access to an intracellular site that is permissive for bacterial survival and proliferation. This was especially evident at 4h p.I. To confirm the results of the flow cytometry analysis, the number of viable bacteria within infected macrophages was determined at progressing times after infection by plating serial dilutions of lysed macrophages on blood agar plates using a gentamicin/lysostaphin protection assay. As shown in Fig. 21, despite the ability of macrophages to kill a substantial proportion of internalized staphylococci at 2h p.I., a proportion of internalized bacteria was able to survive intracellularly. Interestingly, a decrease in bacterial killing rates from 42.3 ± 8.5 % to 27.9 ± 12.6 % was observed between 2h and 4h p.I. (Fig. 21). Since all extracellularly remaining, non-internalized bacteria were killed by lysostaphin treatment at 0h p.I., the increase in bacterial burden during this period can only arise from intracellular replication of *S. aureus*. The actively replicating *S. aureus* may cause macrophage cell death and get released into the extracellular milieu where they are killed by gentamycin or get phagocytosed by neighboring macrophages. In this regard, several studies have reported that macrophages harboring live *S. aureus* are killed by the replicating intracellular bacteria within hours after phagocytosis[127,363,364]. However, it is also possible that the antimicrobial mechanisms of macrophages are able to control the proliferating bacteria after 4h of infection. At 24h p.I., only a small proportion of 13.3 ± 12.6 % remain viable within the macrophages. These findings supported what was already been shown by FACS analysis.

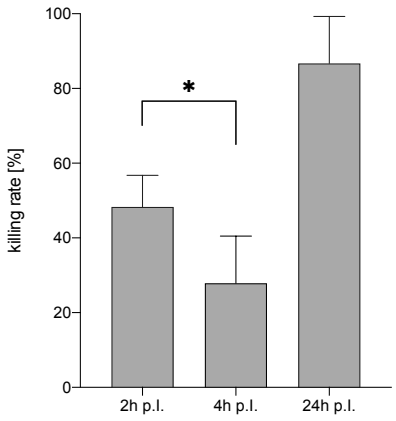

Figure 21 | Killing rates of *S. aureus* pKikume by macrophages at 2h, 4h and 24h p.I.
Intracellular killing activity of macrophages after 2h, 4h and 24h p.I. with *S. aureus* pKikume, displayed as the percentage of killed bacteria in respect to the initial amount of phagocytosed bacteria (0h p.I.). Each bar represents the average ± SD of biological triplicates (n=3, paired t- test, * *p*-value < 0.05).

4.5.1 Intracellular survival of *S. aureus* within macrophages is linked to its capacity to internalize via host cell integrins

The uptake of pathogens into a professional phagocyte is usually mediated by cell surface receptors that can strongly influence the fate of the internalized microorganisms[365]. In this regard, $\alpha_5\beta_1$ integrin signaling is exploited by several microbial pathogens, including *S. aureus,* for entry into host cells. *S. aureus* binds to $\alpha_5\beta_1$ integrin cluster on the surface of host cells indirectly via fibronectin[366–372]. Fibronectin then acts as a molecular bridge linking the $\alpha_5\beta_1$ integrin on the host cell and fibronectin-binding proteins expressed by *S. aureus*[373]. This interaction enables *S. aureus* to enter the host cell. To determine whether different internalization pathways in macrophages could influence the intracellular survival of *S. aureus* pKikume, the effect of blocking the integrin-mediated uptake pathway by either an anti-β1-integrin antibody or a small peptide (RGD) comprising the specific binding motif of fibronectin that engages the integrin $\alpha_5\beta_1$ in the bacterial intracellular survival was tested. The results clearly demonstrated that blocking the $\alpha_5\beta_1$ integrin internalization pathway prevented the intracellular replication of *S. aureus* in macrophages observed between 2h and 4h p.I., resulting in enhanced bacterial killing. A shift from intracellular bacterial replication in untreated macrophages to bacterial killing rates of 19.0 ± 13.6 % for RGD treatment and 17.3 ± 12.8 % for anti-β1-integrin antibody treatment was observed (Fig. 22). Neither the treatment with an isotype control antibody nor a reverse sequence RGD peptide (RGD rev.) affected the intracellular replication of *S. aureus* within macrophages, confirming the specificity of the inhibitors since no change to the untreated cells were observed. Another mechanism used by some microorganisms, for instance streptococci, to invade host cells is to utilize caveolae as a shuttle[374,375]. To examine the impact of caveolar endocytosis on intracellular survival and replication of *S. aureus* within macrophages, methyl-beta-cyclodextrin (MβCD), a known disruptor of caveolae formation[376–378], was used to block this pathway. In contrast to anti-β1-integrin, caveolae disruption did not affect intracellular replication, as indicated by similar replication activities as untreated macrophages (Fig. 22).

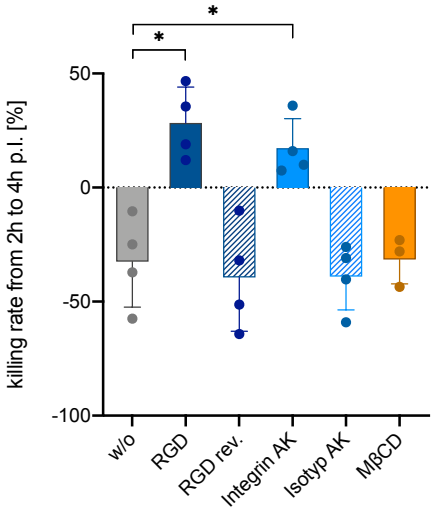

Figure 22 | Killing rates of *S. aureus* pKikume by macrophages between 2h and 4h p.I. after blocking different uptake routes.
Intracellular killing activity of *S. aureus* pKikume by macrophages between 2h and 4h p.I. untreated (grey bar, w/o) or treated with inhibitors to block either integrin-mediated uptake (filled dark blue bar, RGD and filled light blue bar, Integrin AK) or caveosomal uptake (orange bar, MβCD). Negative controls included RGD rev. (patterned dark blue bar) and isotype. Control antibody (patterned light blue bar). Each symbol represents one biological replicate and the bar represents the average ± SD for each treatment (n=3 for MβCD, n=4 for the rest, Brown-Forsythe and Welch ANOVA tests, * $p < 0.05$).

Together these findings indicated that *S. aureus* pKikume engaged $\alpha 5\beta 1$-integrin on the macrophage surface but not caveosomal endocytosis to internalize and gain access to a subcellular compartment that is permissive for intracellular survival and replication.

4.5.2 Examination of *S. aureus* – macrophage interactions by electron and fluorescence microscopy

Field-emission scanning electron microscopy (FESEM) was used to further investigate and visualize the interaction of *S. aureus* pKikume with macrophages. The electron microscope photographs in Fig. 23 show extensive membrane ruffles at the site of *S. aureus* attachment to the surface of macrophages. Membrane ruffling, a mechanism where actin-rich membrane protrusions are formed, has various cellular functions, for instance engaging and responding to pathogens, fluid-phase uptake for nutrient acquisition, or signaling. The induction of cell surface ruffling and macropinocytosis have been reported for pathogens such as *Salmonella typhimurium* and *Legionella pneumophila* to enter host cells[379,380], resulting in the internalization of bacteria into spacious phagosomes[381]. The common organizing structure for both, phagosomes and macropinosomes, is a cup-shaped invagination of the plasma membrane, that form intracellular membrane bound organelles with distinct molecular

mechanisms and signal transduction[381]. Whether the observed membrane ruffles after infection with *S. aureus* pKikume resulted from initial pathogen capture followed by phagocytosis or pathogen induced macropinocytosis remains unclear. On the surface of the infected macrophages no evidence for caveolae formation and bacterial uptake into caveosolae was found, confirming the results of the experiments performed in the previous section.

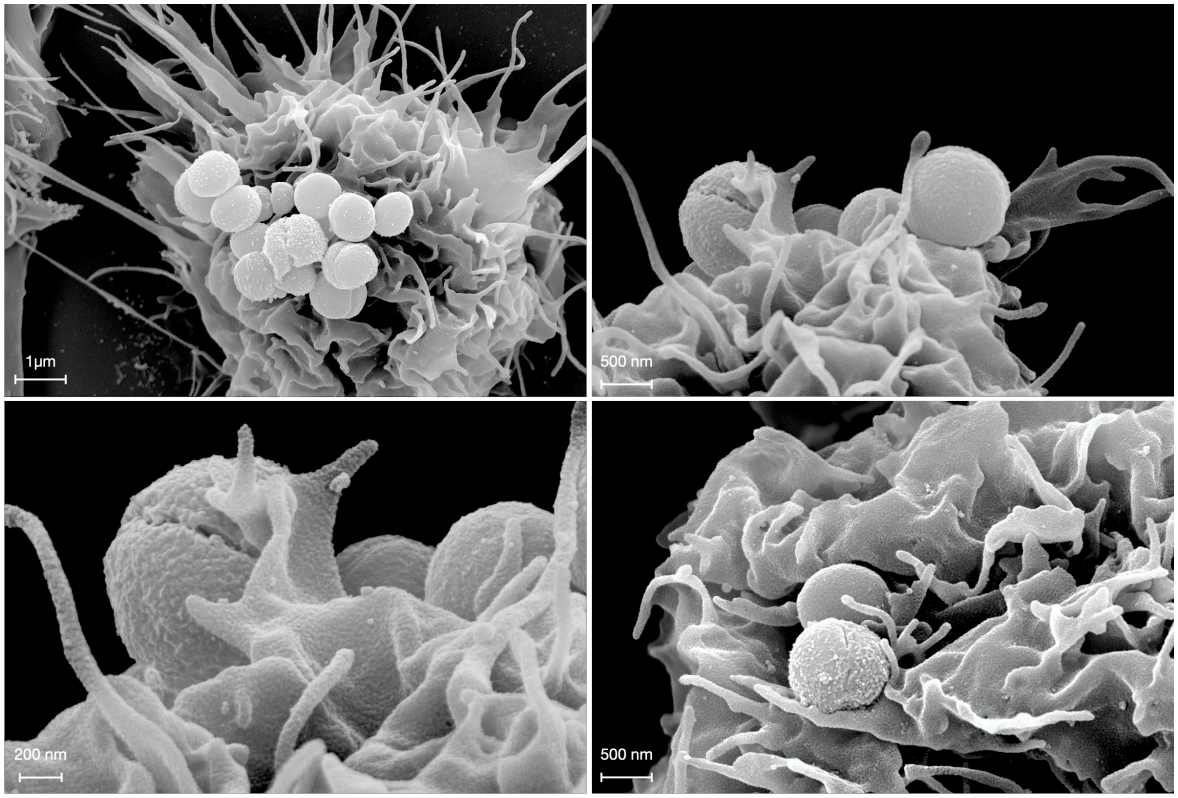

Figure 23 | Field-emission scanning electron microscopy (FESEM) of *S. aureus* pKikume-infected macrophages.
Representative FESEM images of macrophages infected with *S. aureus* pKikume for 2h showing membrane ruffling.

While some studies have reported that *S. aureus* survives within eukaryotic cells by escaping the phagocytic vacuole, gaining access into the host cell cytoplasm[145,287], other studies however have shown that viable *S. aureus* resides within mature phagosomes[363] or within phagosomes that fail to acidify and does not undergo lysosomal fusion[282]. Differences in the experimental settings and/or in the specific strain of *S. aureus* used in the infection assay may explain the observed discrepancies between these studies. To investigate the compartment where intracellular *S. aureus* pKikume is located within macrophages, the cells were pre-loaded with BSA-gold particles, subsequently infected with *S. aureus* pKikume and examined by transmission electron microscopy (TEM) at 0h, 2h and 4h p.I. The TEM photographs depicted in Fig. 24 show fusion of *S. aureus*-containing phagocytic vacuoles with preexisting BSA-gold particles-containing phagosomes (Fig. 24 a) and co-localization of most *S. aureus*

pKikume bacteria with BSA-gold particles in phagolysosomes (Fig. 24 a, c). No evidence of *S. aureus* pKikume escaping from the phagocytic vacuole into the cytoplasm of macrophages was found. Moreover, some intracellular *S. aureus* were found in compartments not yet fused with BSA-gold particles-containing phagosomes (Fig. 24 b, d). These results demonstrate that multiple processes happen simultaneously within macrophages and that with this method regular phagocytosis cannot be clearly distinguished from a pathogen-induced mechanism.

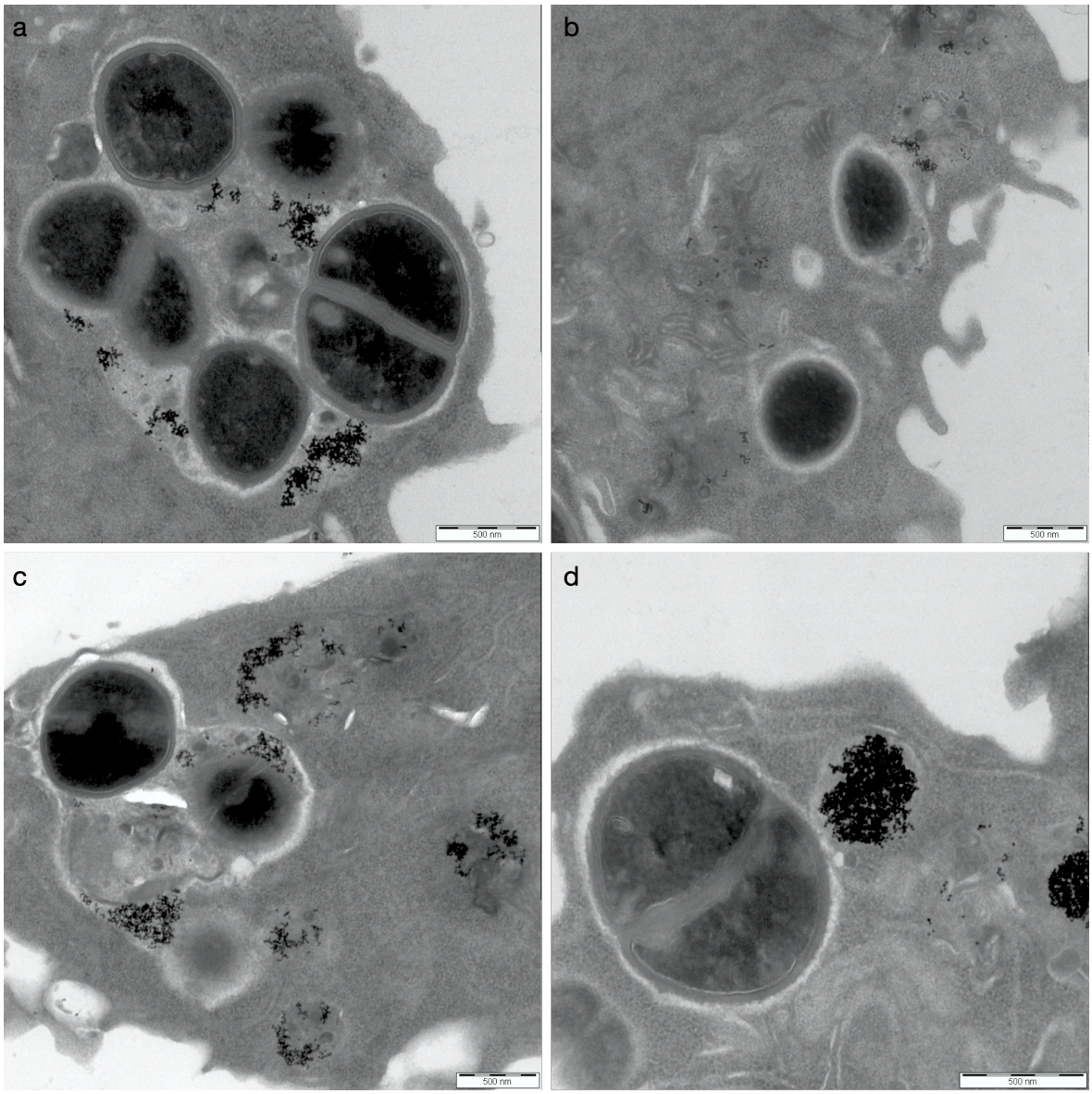

Figure 24 | Transmission electron microscopy (TEM) of *S. aureus* pKikume-infected macrophages.
Representative TEM photographs of *S. aureus* pKikume infected macrophages at 0h (a, b), 2h (c) and 4h p.I. (d). Macrophages were pre-loaded with BSA-gold particles prior to the infection to label phagocytic compartments.

Confocal laser scanning microscopy (CLSM) was used to determine if the intracellular location of metabolically active/proliferating green *S. aureus* pKikume differed from that of

metabolically inactive red *S. aureus* pKikume. For this purpose, macrophages were pre-loaded with a fluorescence marker that labels the endocytic compartments, subsequently infected with *S. aureus* pKikume bacteria non-photoconverted or photoconverted 30 min before acquisition and examined by CLSM as well as by differential interference contrast (DIC) microscopy. Microscopy photographs of macrophages infected with non-photoconverted *S. aureus* (green) depicted in Fig. 25 show co-localization of green-fluorescent *S. aureus* with the labeled endocytic compartments (pink), indicating phagocytosis (white arrows). However, clusters of green-fluorescence *S. aureus* were also found within spacious vacuoles that were not associated with endosomal compartments and most probably contained actively replicating bacteria (Fig. 25 red arrow).

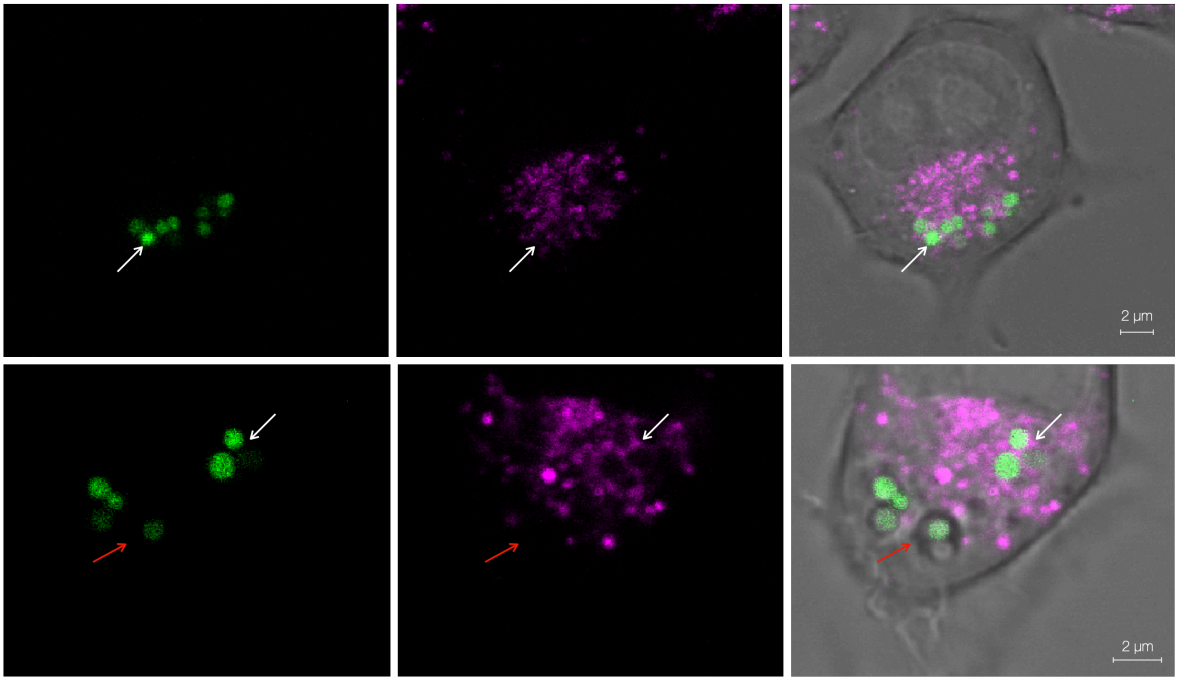

Figure 25 | Confocal laser scanning microscopy (CLSM) images of *S. aureus* pKikume-infected macrophages without photoconversion.
Endocytic compartments of macrophages were labeled using Alexa 647 conjugated dextran beads (pink) one day before infection with *S. aureus* pKikume (green). Single green (mKikume Green) and pink (endosomal marker) fluorescence channels are merged with the DIC channel and shown as overlay on the right. Scale bar, 2 µm.

Macrophages pre-loaded with the endocytic compartment marker were infected with *S. aureus* pKikume and photoconverted 30 min prior to microscopy image acquisition to examine the compartments were metabolically active/proliferating (green fluorescent) and metabolically inactive (red fluorescent) bacteria are located. Microscopy photographs depicted in Fig. 26 (red arrow) show green proliferating bacteria within spacious vacuole that did not co-localize with endocytic compartments (pink). *S. aureus* pKikume bacteria that did not fully

recover the green fluorescence due to low metabolic activity inside the macrophage and therefore exhibited a yellow color, were observed in close proximity to endocytic compartments (Fig. 27 white arrow).

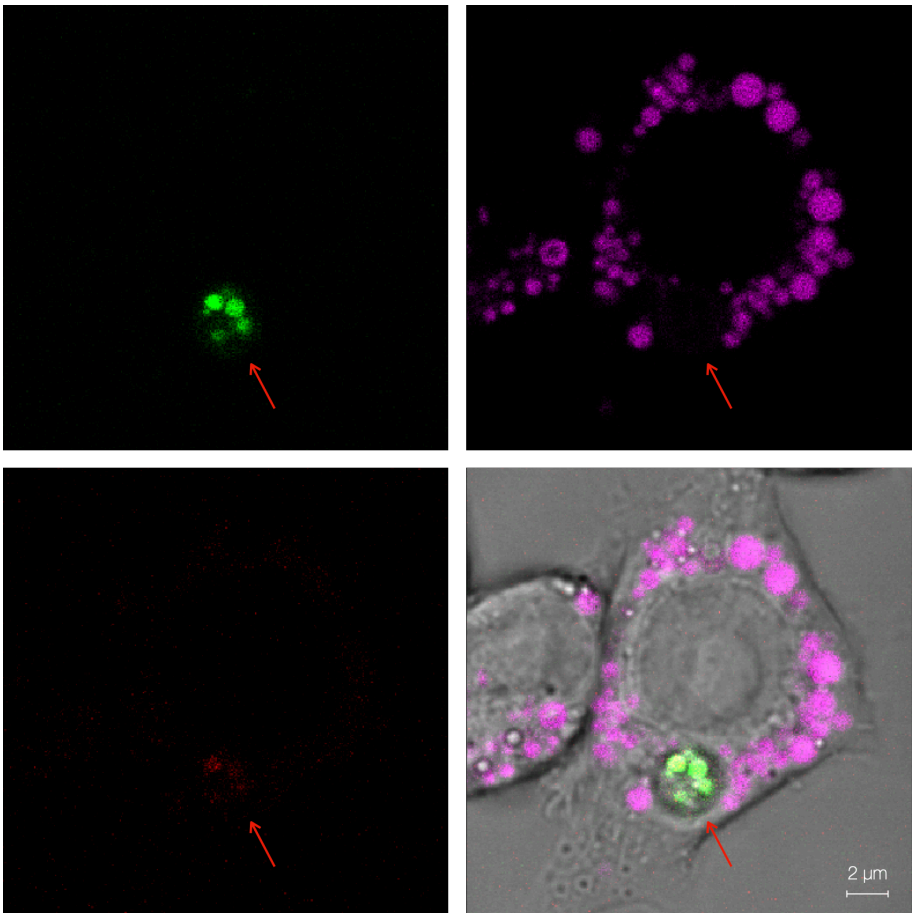

Figure 26 | Confocal laser scanning microscopy (CLSM) images of macrophages infected with photoconverted *S. aureus* pKikume.
Endocytic compartments of macrophages were labeled using Alexa 647 conjugated dextran beads (pink) one day before infection with *S. aureus* pKikume for 2h. 30 min before imaging, infected cells were photoconverted. Single green (mKikume Green), red (mKikume red) and pink (endosomal marker) fluorescence channels are merged with the DIC channel and shown as overlay on the right bottom. Scale bar, 2 µm.

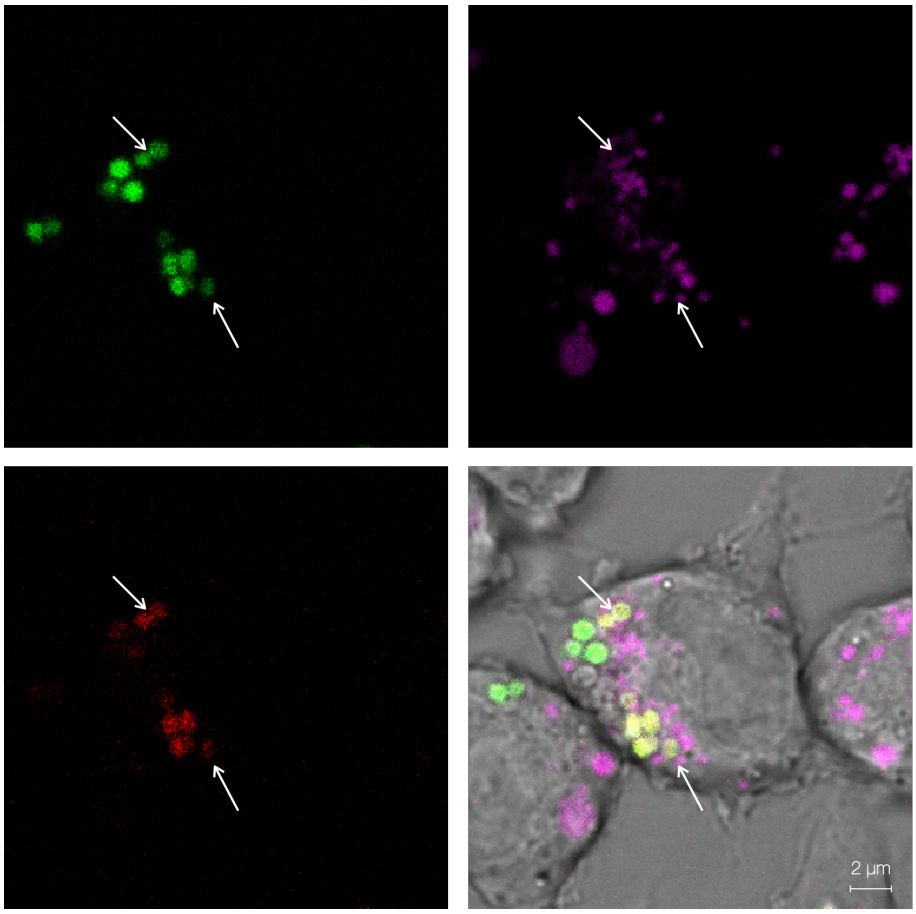

Figure 27 | Confocal laser scanning microscopy (CLSM) images of macrophages infected with photoconverted *S. aureus* pKikume.
Endocytic compartments of macrophages were labeled using Alexa 647 conjugated dextran beads (pink) one day before infection with *S. aureus* pKikume for 2h. 30 min before imaging, infected cells were photoconverted. Single green (mKikume Green), red (mKikume red) and pink (endosomal marker) fluorescence channels are merged with the DIC channel and shown as overlay on the right bottom. Scale bar, 2 µm.

Taken together, the results of the different microscopy techniques cannot clearly answer the question about the intracellular site of *S. aureus* pKikume replication. As professional phagocytes, macrophages are quite active cells with highly dynamic processes happening at the same time and a fast cell membrane turnover[212,382,383]. Nevertheless, the distinct spacious vacuoles seen in Fig. 26 containing metabolically active bacteria give evidence for a mechanism where enclosed *S. aureus* are able to replicate within a host cell compartment that does not fuse with endosomal compartments.

4.6 Intracellular survival of *S. aureus* pKikume within M1 and M2 macrophages

4.6.1 Polarization of bone marrow derived macrophages into M1 or M2 phenotypes

As part of the immune system, macrophages infiltrate the site of infection and subsequently are exposed to different microenvironmental signals that can promote their polarization status into a variety of macrophage phenotypes with distinct functions. Thus, while the presence of Th1-related cytokines such as IFN-γ or TNF-α promotes M1 polarization, Th2-related cytokines such as IL-4 or IL-10 induces M2 polarization[384]. M1 macrophages are pro-inflammatory and can kill intracellular bacteria much more efficiently than anti-inflammatory M2 macrophages, which are mainly involved in tissue repair processes[385]. Therefore, the balance between M1- and M2-polarized macrophages at the site of infection can influence pathogen clearance and can represent varied reservoirs for intracellular pathogen survival and/or replication.

To investigate the influence of macrophage polarization on the capacity of *S. aureus* to survive and replicate within these professional phagocytes, murine bone marrow derived macrophages (BMDM) were polarized into M1 or M2 macrophages using different growth factors as described in the Materials and Methods section. To validate the polarization status, RNA was extracted from both macrophage subsets, and gene expression patterns of known M1 or M2 marker were investigated by qRT-PCR. The increased expression of the cell activation marker CD69 as well as pro-inflammatory cytokines (IL-6, IL-1β, and TNFα) confirmed the M1 macrophage phenotype whereas the increased expression of classically M2-associated factors like arginase 1 and PPARγ confirmed the M2 phenotype (Fig. 28).

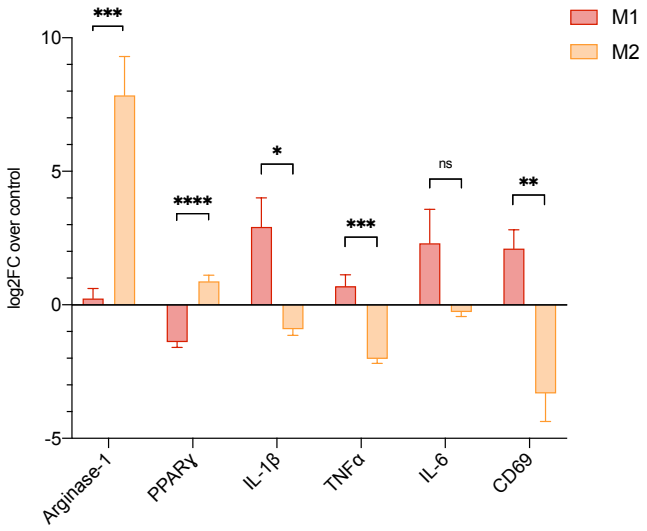

Figure 28 | Gene expression analysis of specific marker genes for M1 and M2 macrophages using qRT-PCR.
Different polarization status of M1 and M2 macrophages were validated by comparing the fold changes [log_2] in expression of selected genes in M1 (red bars) and M2 (orange bars) macrophages compared to unstimulated BMDMs using qRT-PCR (n=6, multiple t-tests, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

The gene expression data was then validated at the protein level by FACS analysis of cell surface receptors as well as intracellular arginase 1. Staining of specific macrophage cell surface markers included the general monocyte marker F4/80 and CD11b as well as CD11c for M1 polarized macrophages and CD206 for M2 macrophages. The expression of CD11c and CD206 in M1- and M2-polarized macrophages (Fig. 29 b) was determined in the macrophage population gated according to the expression of F4/80 and CD11b (Fig. 29 a). The representative FACS plots in Fig. 29 confirm the successful polarization of M1 macrophages and M2 macrophages.

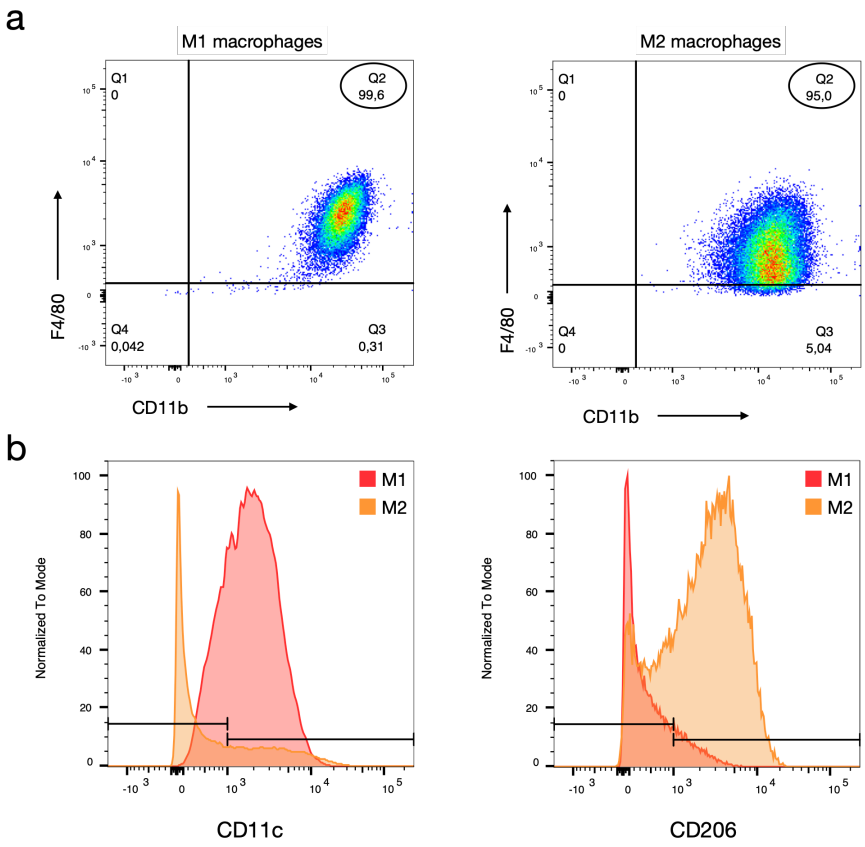

Figure 29 | FACS analysis of cell surface receptors of M1- and M2-polarized macrophages.
FACS plots from a representative experiment of M1- or M2 polarized macrophages with cell surface receptor staining of the general monocyte marker F4/80 and CD11b (a), and CD11c as M1 marker and CD206 as M2 marker (b). Dot plots (a) illustrate gating of monocytes according to F4/80 and CD11b expression; Histograms (b) illustrate the normalized percentage of CD11c or CD206 expressing cells in the monocyte subset (Q2).

To further corroborate the phenotype of M1 and M2 macrophages, staining of the cell surface markers F4/80 and CD11b followed by intracellular staining of arginase 1 was performed. Arginase 1 was predominantly detected within the F4/80+CD11b+ population of

M2 polarized macrophages, as shown by representative FACS plots (Fig. 30 a and b) and in absolute numbers (Fig. 30 c).

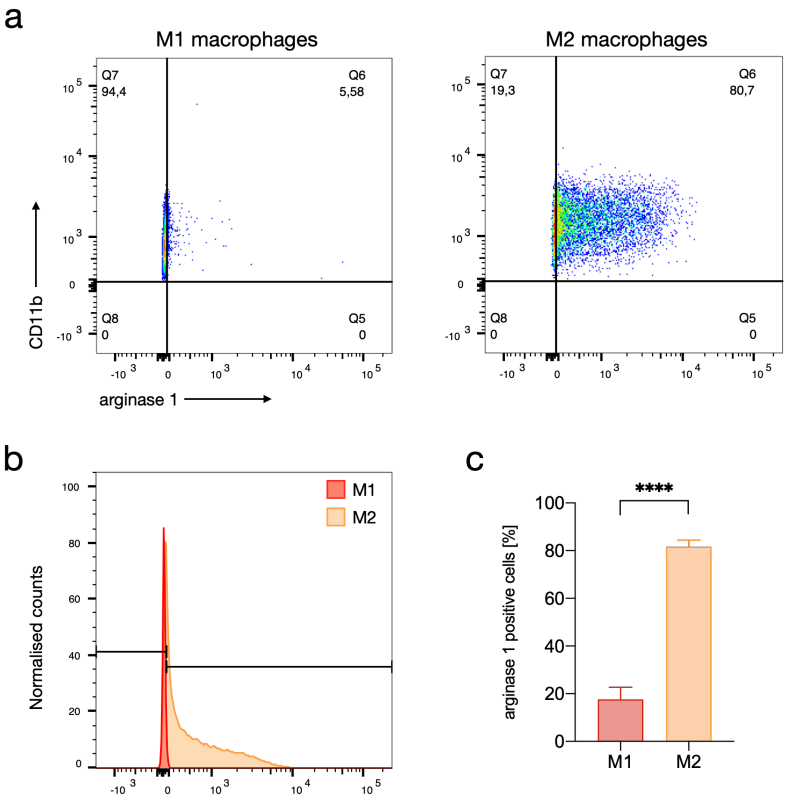

Figure 30 | FACS analysis of intracellular arginase 1 in M1- and M2- polarized macrophages.
(a) FACS dot plots from representative experiments of intracellular arginase 1 staining of M1- and M2-polqarized macrophages after staining the cell surface monocyte marker F4/80 and CD11b. (b) Histogram overlay of a representative arginase 1 stain in M1 and M2 macrophage. (c) Quantification of arginase 1 positive cells within M1- and M2- polarized macrophages (n=12, Welch's t-test, **** $p < 0.0001$).

Besides the expression of specific markers, the polarization of BMDM's into M1 or M2 macrophages also increases the cell size[386], as shown for a representative FACS experiment in Fig. 31. A clear shift from smaller BMDM's (grey curve) to increased cell sizes for activated M1 (orange curve) and M2 (red curve) was observed (Fig. 31 a). Depicted in Fig. 31 b, the mean relative cell sizes increase after activation of BMDM's.

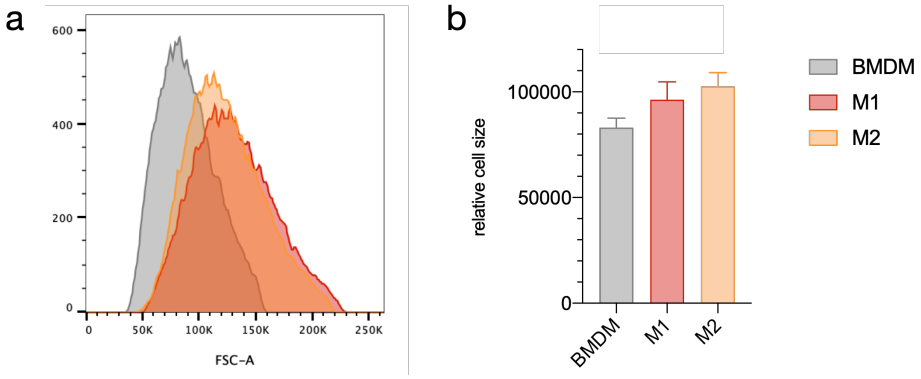

Figure 31 | FACS analysis of increased cell size after activation of BMDM's into M1 or M2 macrophages.
Upon activation the cell size of differentiated BMDM's increase after activation into M1- or M2-polarized macrophages shown by a representative FACS histogram (a) and a quantitative analysis (n=5).

Taken together, the results demonstrate a successful polarization of BMDM's into M1 or M2 macrophages with approved features for each subset on different molecular levels and with several methods.

4.6.2 M2-polarized macrophages are more permissive for *S. aureus* pKikume intracellular survival than M1-polarized macrophages

To determine if macrophages with different polarization status differed in their capacity to kill intracellular *S. aureus* pKikume, M1 and M2 macrophages were generated *in vitro* as described above, infected with *S. aureus* pKikume and viable intracellular bacteria were determined at progressing times after infection. M1 macrophages were capable to efficiently control intracellular *S. aureus* as indicated by a steady increase in killing rates over time (Fig. 32). In contrast, M2 macrophages were unable to restrict intracellular *S. aureus* proliferation, as the killing rates decline over time, resulting in progressive increase in intracellular bacterial loads (Fig. 32). Thus, at 24h p.I., a remarkable difference in the killing rate of intracellular *S. aureus* pKikume was observed between M1 and M2 macrophages, with 59.7% ± 23.1 % intracellular bacteria reduction in respect to the initial number of intracellular bacteria in M1 macrophages and 1.6 ± 25.1 % intracellular bacteria reduction in M2 macrophages.

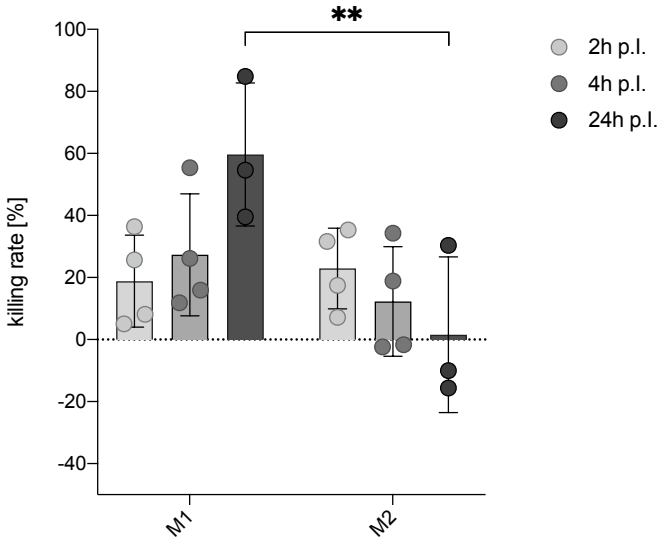

Figure 32 | Killing rates of *S. aureus* pKikume by M1- and M2-polarized macrophages at 2h, 4h and 24h p.I.
Intracellular killing activity of *S. aureus* pKikume by M1 and M2 macrophages after 2h, 4h and 24h p.I. is displayed as the percentage of initially phagocytosed bacteria (0h p.I.). Each symbol represents one biological replicate; each bar represents the average ± SD of biological triplicates (n=3 for 24h p.I., n=4 for 2 and 4h p.I., Sidak multiple comparisons test, ** *p*-value < 0.01).

To investigate the metabolic status of *S. aureus* pKikume within M1- and M2- polarized macrophages, FACS analysis of infected M1 and M2 macrophages were performed at 0h, 2h, 4h and 24h p.I. with photoconversion of the intracellular bacteria 30 min before sampling. The bar chart in Fig. 33 illustrates the proportion of infected cells harboring metabolically active green fluorescent *S. aureus* pKikume. The percentage of infected macrophages containing active intracellular green bacteria was similar between M1 and M2 macrophages at 0h p.I. and was higher in infected M2 macrophages than in M1 macrophages at 2h, 4h and 24h p.I., although statistical significance was only reached at 24h p.I.

This result corroborates what was already found in the experiments assessing intracellular bacterial viability by plating, suggesting that M1 macrophages exhibit a more hostile environment for intracellular survival/replication of *S. aureus* pKikume indicated by a higher killing activity (Fig. 32) of the host cell and a lower metabolic activity of the intracellular bacteria (Fig. 33) both statistically significant at 24h p.I.

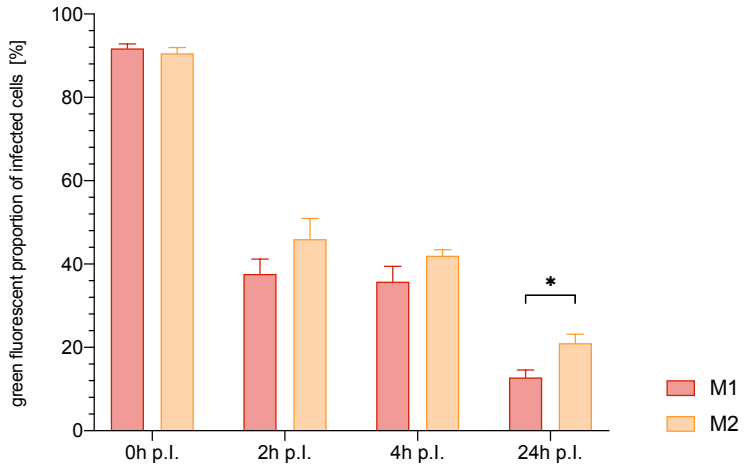

Figure 33 | Proportion of infected M1 and M2 macrophage cells that harbor metabolic active *S. aureus* pKikume over the course of infection.
Percentage of M1- (red bars) and M2-polarized (orange bars) macrophages harboring green fluorescent *S. aureus* pKikume at 0h, 2h, 4h and 24h p.I. within the total infected population determined by flow cytometry analysis. Each bar represents the mean ± SD of 6 replicates (Welch's t- test, * p-value < 0.05).

In order to maintain the M2 macrophage phenotype throughout the infection, the specific growth factors for M2 polarization were kept during the whole experiment. However, as shown in Fig. 34, the M2 subset changed its gene expression pattern 4h and 24h p.I. into a more M1-like, pro-inflammatory pattern (light and dark grey bars) compared to their uninfected M2 macrophage expression profile (white bars) in response to the *S. aureus* infection. This change was significant for the cytokines IL-1β and IL-6. Nevertheless, the M1 macrophages overall showed higher gene expression levels of the pro-inflammatory factors (IL-1β, TNFα, and IL-6) than the M2 subset, suggesting a delay in response to the pathogen due to the different polarization status at the beginning of the infection.

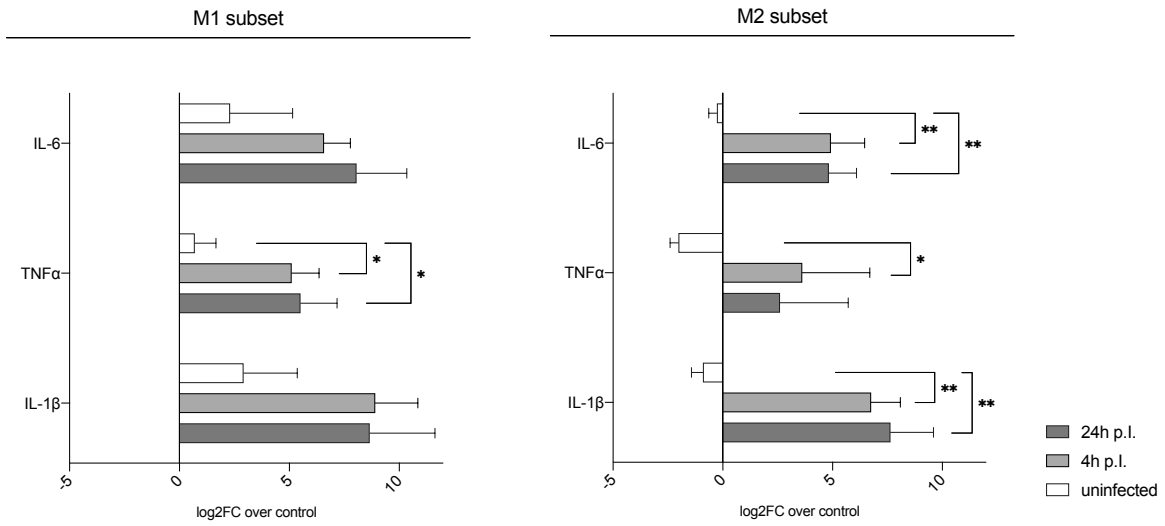

Figure 34 | Gene expression analysis of specific proinflammatory genes for M1 and M2 macrophages before and after infection with *S. aureus* pKikume using qRT-PCR.
Different polarization status of M1 and M2 macrophages was determined before (white bars), 4h p.I. (light grey bars) and 24h p.I. (dark grey bars) with *S. aureus* pKikume by qRT-PCR. The bars represent the average ± SD fold changes [log_2] in expression of selected genes in M1 (left panel) and M2 (right panel) macrophages compared to unstimulated BMDMs (n=6, Holm-Sidak multiple t-test, * $p < 0.05$, ** $p < 0.01$).

4.7 Intracellular replication of *S. aureus* pKikume in non-professional phagocytes

Besides macrophages, *S. aureus* is capable to invade non-professional phagocytic cells such as epithelial cells[296,387]. These facultative phagocytes have an important function in removing dead cells from the epithelium to maintain membrane integrity but they have a much more limited capacity to kill internalized pathogens than professional phagocytes. Whereas survival of *S. aureus* within phagocytic cells has been proposed as mechanism used by *S. aureus* to disseminate within the host and use the phagocytic cells as shuttle to penetrate into deeper tissue[5,343], intracellular survival within non-phagocytic cells may protect *S. aureus* from extracellular host defense mechanisms and enable the bacteria to persist within the host[388,389].

To determine the capacity of *S. aureus* pKikume to internalize and survive within non-phagocytic cells, human epithelial HEp-2 cells were infected with *S. aureus* pKikume and viable intracellular bacteria were determined at progressing times after infection by plating cell lysates on blood agar plates. As shown in Fig. 35, *S. aureus* pKikume was capable to efficiently internalize within HEp-2 cells and replicate, indicated by the increase of intracellular bacteria from $4.9x10^5 \pm 3.5 x10^5$ CFU/ml to $7.4x10^5 \pm 4.4x10^5$ CFU/ml between 2h and 4h p.I (Fig. 35 a). This finding is in line with the results seen in immortalized macrophages (IMΦ) and M2 macrophages showing the proliferation of intracellular *S. aureus* pKikume between 2h and 4h p.I. (Fig. 35 b). At 24h p.I. the bacterial burden dropped to $1.7x10^5 \pm 9.7x10^4$ CFU/ml (Fig. 35 a).

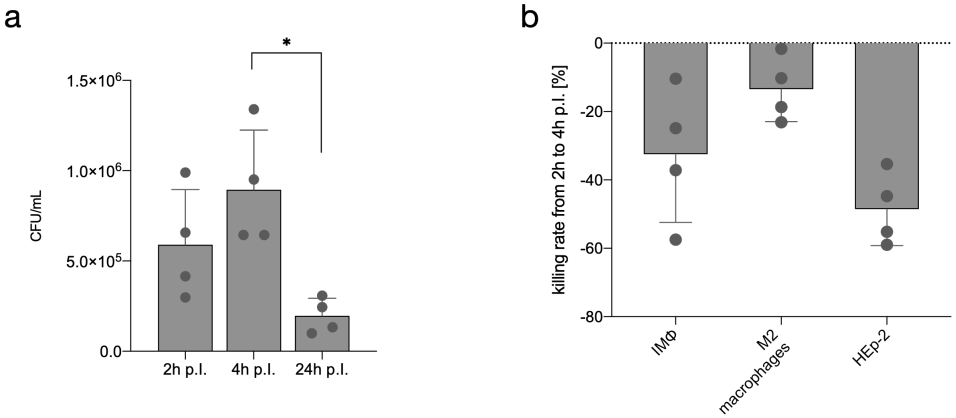

Figure 35 | Intracellular burden of *S. aureus* pKikume within HEp-2 epithelial cells over the course of infection.
(a) Number of viable intracellular bacteria after infection of HEp-2 cells with *S. aureus* pKikume at 2h, 4h and 24h p.I. displayed as CFU/ml (n=4, Brown-Forsythe and Welch ANOVA tests, * $p < 0.05$). (b) Percentage of *S. aureus* pKikume intracellular killing by HEp-2 cells compared to immortalized macrophages (IMΦ) and M2-polarized macrophages between 2h and 4h p.I. with (n=4).

4.7.1 Intracellular replication of *S. aureus* pKikume in human and murine epithelial cells is dependent on caveolae formation

Because internalization via β1-integrin on the host cell surface seems to play a crucial role for *S. aureus* survival within macrophages (see Fig. 22), the use of this pathway for *S. aureus* to survive within epithelial HEp-2 cells, a non-professional phagocytic cell type, was investigated. However, in contrast to what has been observed in macrophages, antibody blockage of β1-integrin did not influence the survival of intracellular *S. aureus* since the killing rate between 2h and 4h p.I. was similar between antibody-treated and untreated HEP-2 cells and intracellular replication still occurred (Fig. 36). Caveolae and/or lipid rafts constitutes an additional pathway that has been implicated in the intracellular survival of bacteria within epithelial cells[375,390,391]. To determine the role of caveolae in the survival of *S. aureus* within HEp-2 cells, caveolae formation was inhibited by pre-incubation of HEp-2 cells with methyl-β-cyclodextrin (MβCD), a known disruptor of caveolae formation through depletion of cholesterol in lipid rafts, followed by *S. aureus* infection. As shown in Fig. 36, inhibition of caveolae resulted in significant inhibition of intracellular bacterial replication and increased bacterial killing to 36.3 ± 25.6 % between 2h and 4h p.I. These results were further corroborated by pre-treating the HEp-2 cells with Filipin III, an agent disrupting the structural integrity of caveolae, which resulted in increased bacterial killing rates of 18.2 ± 11.3 % (Fig. 36).

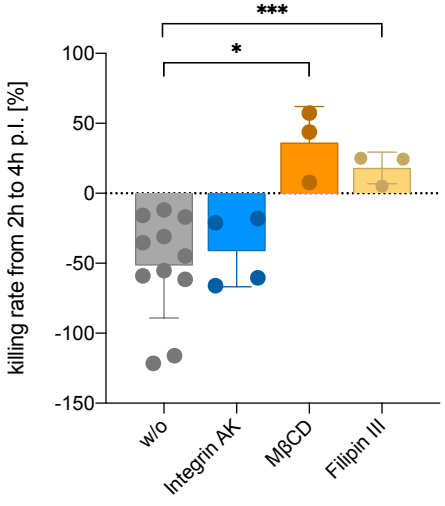

Figure 36 | Killing of *S. aureus* pKikume by HEp-2 between 2h and 4h p.I. in the presence or absence of different inhibitors.
Intracellular killing rates of *S. aureus* pKikume by HEp-2 cells between 2h and 4h p.I. without treatment (grey bar, w/o) or treated with inhibitors to either block integrin mediated uptake (blue bar, Integrin AK) or caveosomal uptake (orange and yellow bar, MβCD and Filipin III). Each symbol represents the killing rate in one biological replicate, each bar represents the average ± SD for each treatment (Brown-Forsythe and Welch ANOVA tests, * $p < 0.05$, *** $p < 0.001$).

Taken together, these results demonstrate that *S. aureus* used the caveolar pathway to survive and replicate within HEp-2 epithelial cells. Therefore, it seems that the pathway engaged by *S. aureus* to internalize and survive intracellularly is highly dependent on the cell type. While *S. aureus* pKikume uses β1-integrin in macrophages to facilitate a successful intracellular lifestyle, in epithelial HEp-2 cells the pathogen uses the caveolarpathway.

To determine if the utilization of caveolae by *S. aureus* pKikume for internalization within epithelial cells was host dependent for human cells, infection experiments were performed using the murine mammary epithelial cell line HC-11. Consistent with the results obtained with human HEp-2 cells, *S. aureus* was also able to replicate within murine HC-11 cells between 2h and 4h p.I. (Fig. 37). Furthermore, blocking caveolae formation by pre-incubation with either MβCD or Filipin III inhibited intracellular bacterial proliferation and enhanced bacterial killing to 51.3 ± 29.6 % and of 68.7 ± 11.8 % (Fig. 37), respectively. These results underline the general relevance of caveolae formation for *S. aureus* pKikume uptake, intracellular survival and proliferation within epithelial cells.

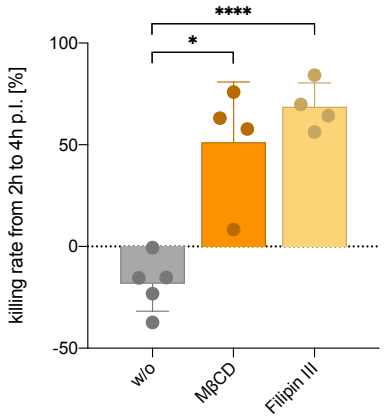

Figure 37 | Killing of *S. aureus* pKikume by HC-11 cells between 2h and 4h p.I. in the presence or absence of caveolae inhibitors.
Intracellular killing rates of *S. aureus* pKikume by HC-11 cells between 2h and 4h p.I. without treatment (grey bar, w/o) or treated with inhibitors to block caveosomal uptake (orange and yellow bar, MβCD and Filipin III). Each symbol represents the killing rate in one biological replicate, each bar represents the average ± SD for each treatment (Brown-Forsythe and Welch ANOVA tests, * $p < 0.05$, **** $p < 0.0001$).

Field-emission scanning electron microscopy (FESEM) was further used to visualize the internalization of *S. aureus* pKikume in HEp-2 cells. Electron microscope photographs depicted in Fig. 38 show that, in addition to membrane protrusions and ruffles (Fig. 38 a), *S. aureus* bacteria were mainly found in cavity-like invaginations on the surface of the epithelial cells (Fig. 38 b-d). In HEp-2 cells where caveolae formation has been disrupted by pre-

treatment with MβCD, single bacteria were located in indented small pits that did not resemble the large cavity-like invaginations typical of caveolae (Fig. 38 e) and uptake of bacterial aggregates was impaired and bacteria remained attached to the epithelial cell surface (Fig. 38 f).

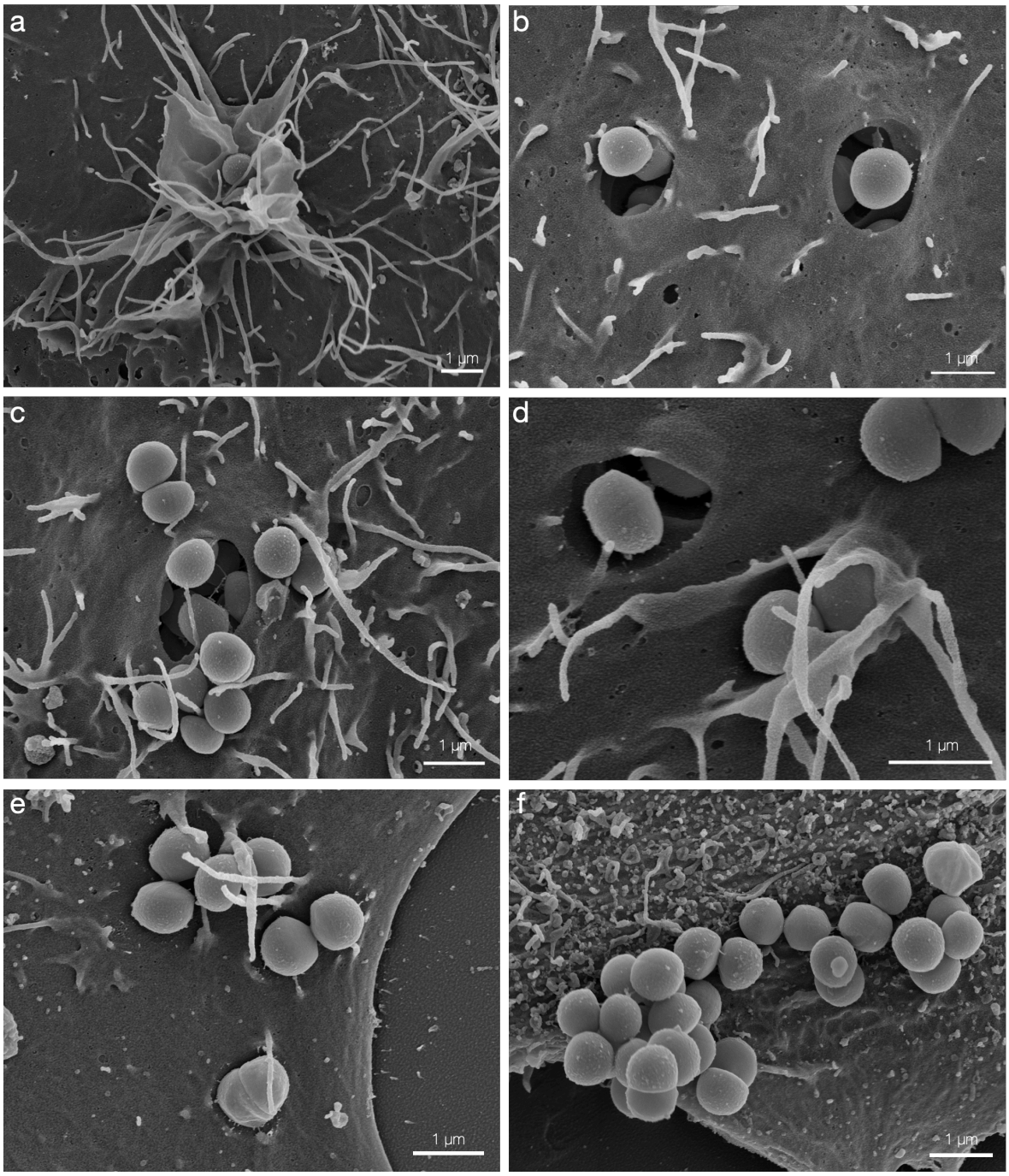

Figure 38 | Field-emission scanning electron microscopy (FESEM) of *S. aureus* pKikume infected HEp-2 cells.
Field-emission scanning electron microscopy (FESEM) images of un-treated HEp-2 cells infected with *S. aureus* pKikume for 30 min (a) and 2h (b-d) as well as MβCD pre-treated HEp-2 cells infected for 2h with *S. aureus* pKikume (e, f). Scale bar, 1 µm.

Transmission electron microscopy (TEM) analysis of HEp-2 cells, which were pre-loaded with BSA-gold particles followed by infection with *S. aureus,* mainly shows no co-localization of the endosomes loaded with BSA-gold particles and the intracellular compartment where *S. aureus* was located at 0h (Fig. 39 a, b) and 4h p.I. (Fig. 39 c, d). This indicates a potential intracellular location which is independent of the classical endosomal pathway. Although most intracellular bacteria resided in a membrane-bound compartment (Fig. 39 b, white arrow), a few of them might be located free in the cytosol of the host epithelial cell (Fig. 39 c, white arrow). Nevertheless, this has to be proven by further studies. The data obtained in this thesis clearly demonstrate, that the intracellular niche of *S. aureus* in epithelial cells is diverse, ranging from compartments of the endosomal pathway to compartments with unknown features or even free in the host cell cytosol.

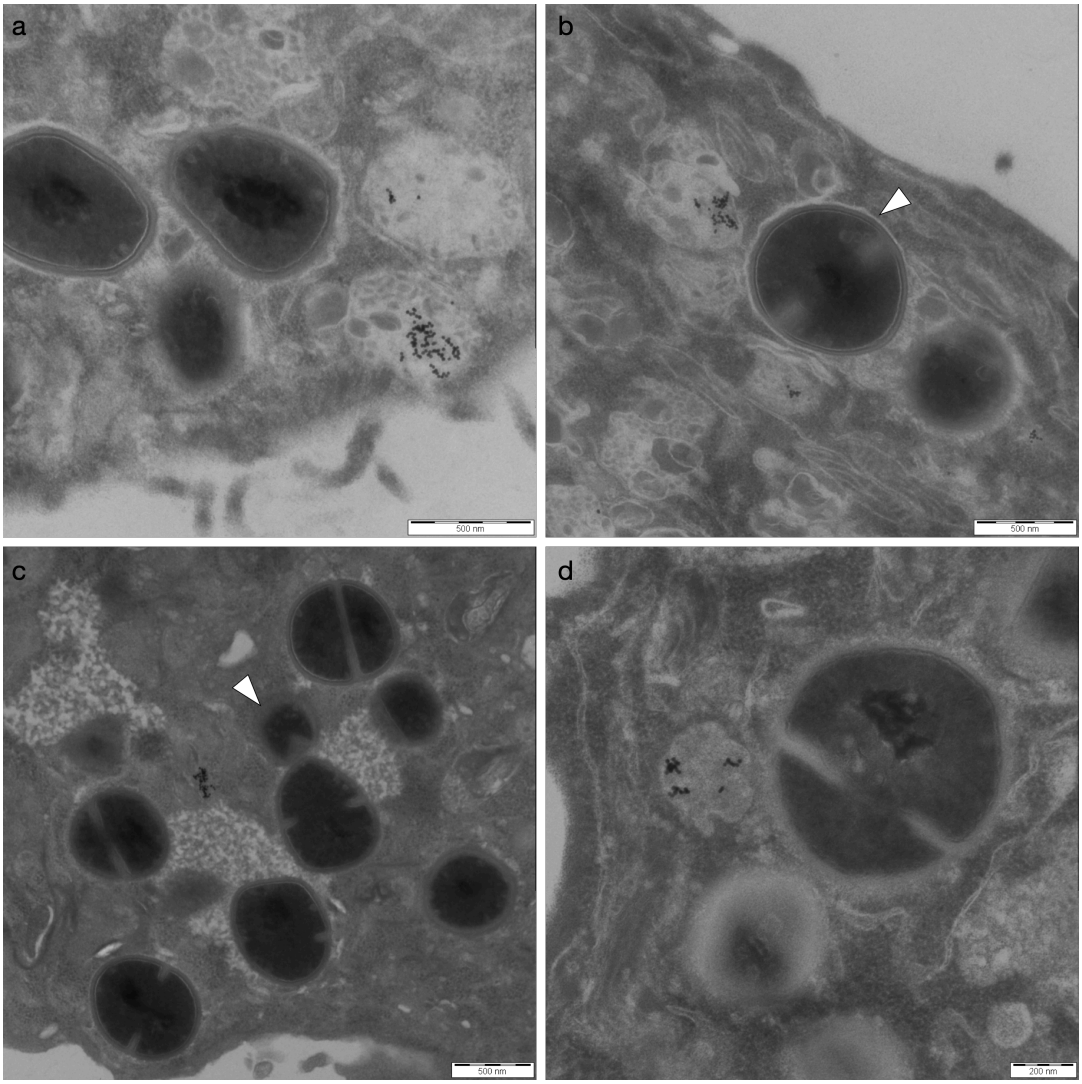

Figure 39 | Transmission electron microscopy (TEM) of *S. aureus* pKikume-infected epithelial HEp-2 cells.
Representative TEM photographs of *S. aureus* pKikume infected epithelial HEp-2 cells at 0h (a, b) and 4h p.I. (c, d). HEp-2 cells were pre-loaded with BSA-gold particles prior to the infection to label phagocytic compartments.

4.7.2 Identification of a HEp-2 cell subset harboring high proliferating *S. aureus* pKikume

To investigate a potential link between the metabolic status of *S. aureus* pKikume and its capacity to persist and replicate within HEp-2 epithelial cells, infection experiments were performed with photoconversion of internalized bacteria 30min before sampling at 0h, 2h and 4h p.I. followed by flow cytometry analysis. The representative FACS plots in Fig. 40 show a stable proportion of *S. aureus* infected HEp-2 cells over the course of infection with a subset of cells harboring metabolic active, highly replicating green bacteria evolving over time to a distinct population at 4h p.I.

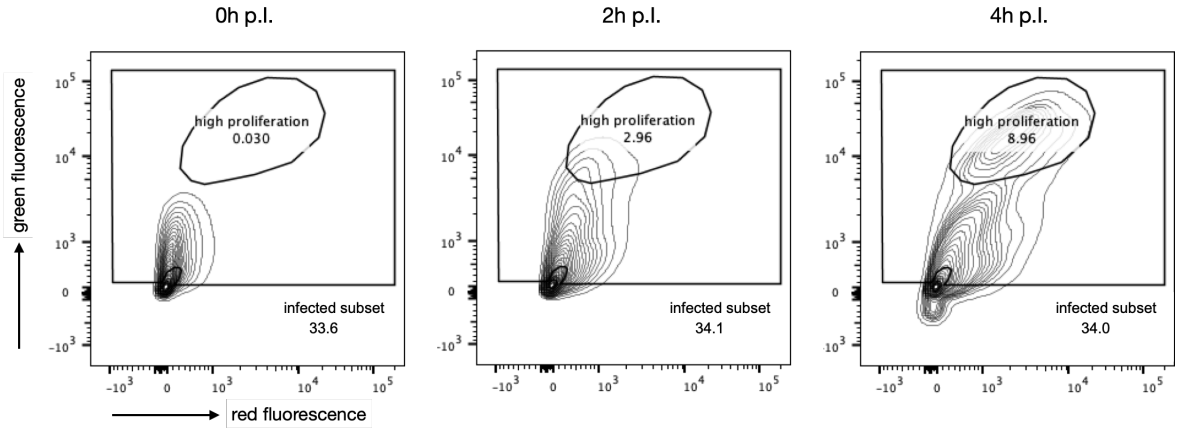

Figure 40 | Flow cytometry analysis of *S. aureus* pKikume-infected HEp-2 epithelial cells.
Contour plots from a representative experiment of *S. aureus* pKikume-infected HEp-2 cells showing the percentage of infected cells within the gate (infected subset) at 0h, 2h and 4h p.I. In each panel, the subpopulation of infected HEp-2 cells harboring metabolic active, highly proliferating green fluorescent bacteria is indicated by the gate 'high proliferation'.

The quantitative analysis of the FACS data depicted in Fig. 41, indicates that the overall percentage of *S. aureus* pKikume-infected HEp-2 cells is constant over time (Fig. 41 a). However, the percentage of HEp-2 cells harboring metabolically active/proliferating bacteria within the infected population increases over the course of infection (Fig. 41 b).

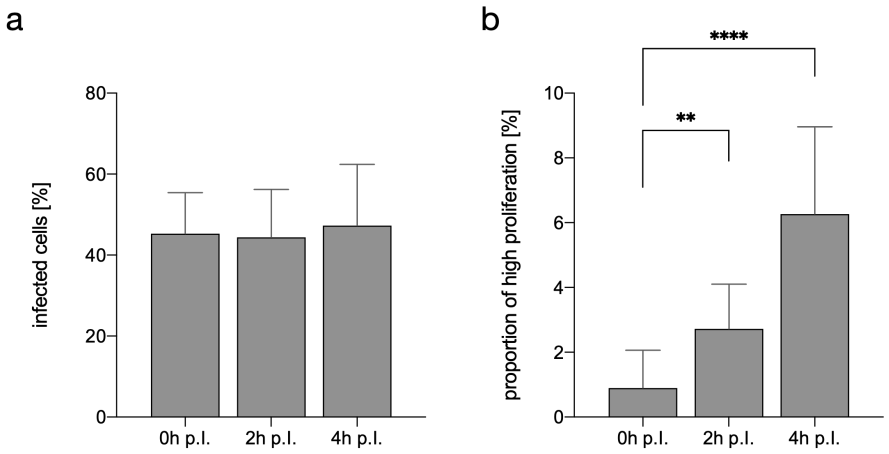

Figure 41 | Proportion of *S. aureus* pKikume-infected HEp-2 cells at 0h, 2h and 4h p.I.
(a) Percentage of *S. aureus* pKikume-infected HEp-2 epithelial cells at 0h, 2h and 4h p.I. determined by flow cytometry. (b) Percentage of infected HEp-2 cells harboring metabolic active/proliferating bacteria within the total population of infected cells at 0h, 2h and 4h p.I. The bars represent the average ± SD of biological replicates (n=11, Brown-Forsythe and Welch ANOVA tests, ** $p < 0.01$, **** p-value < 0.0001).

4.7.3 α-Hemolysin facilitates *S. aureus* internalization into epithelial cells

For the internalization into host cells, pathogens express a variety of surface proteins as well as secreted factors to promote the initial contact to the host which is crucial for bacterial uptake and/or the induction of immunomodulatory signaling cascades. To determine if bacterial factors are expressed by *S. aureus* pKikume in a host cell specific manner, the expression of a subset of genes, that have been previously reported[370,392–397] to facilitate staphylococcal adherence and internalization into various host cell types, was investigated. Therefore, the expression levels of genes encoding fibronectin-binding protein A (*fnbpA*), alpha-toxin (*hla*), the extracellular adherence protein eap/map (*map*) and clumping factor A (*clfA*) were analysed in extracellular bacteria after 2h of co-cultivation with host cells by qRT-PCR.

It could be demonstrated that all investigated genes were up-regulated in *S. aureus* to a similar extent after co-cultivation with either macrophages (professional phagocytes) or epithelial cells (non-professional phagocytes) (Fig. 42), indicating a host cell type independent response of *S. aureus* in the early phase of host cell pathogen interaction.

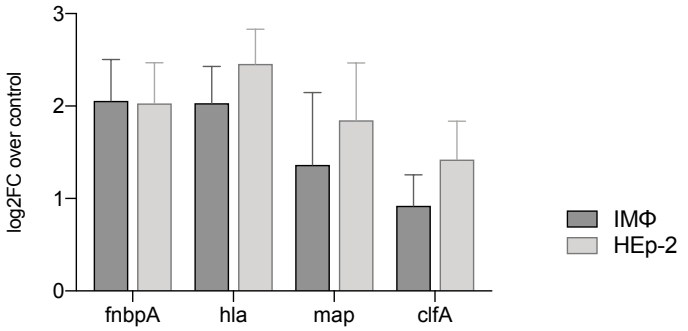

Figure 42 | Expression levels of selected genes encoding virulence factors of *S. aureus* pKikume after co-cultivation with macrophages (professional phagocytes) or HEp-2 cells (non-professional phagocytes).
Expression levels of *fnbpA, hla, map* and *clfA* in *S. aureus* pKikume after 2h co-cultivation with either macrophages (IMΦ) (dark grey bars) or HEp-2 cells (light grey bars) in comparison to the baseline expression levels of the genes in inoculum bacteria (control) determined using qRT-PCR. Data are expressed as fold changes [log_2]. Each bar represents the average of 6 biological replicates ± SEM.

Although no differences in the expression of the investigated adhesins and invasins were observed, that could be claimed responsible for the integrin-mediated uptake favored for professional phagocytes, or the caveolae-mediated uptake favored in non-professional epithelial cells, respectively, the role of staphylococcal toxin hla, known to elicit multiply cellular responses, was further investigated.

From literature, it is known that the staphylococcal alpha-toxin is structurally similar to the cholera toxin secreted by the bacterium *Vibrio cholerae*[398]. Moreover, it has been shown that cholera toxin strongly binds to caveolae and/or lipid rafts followed by internalization [399–402].

As the previous findings of this thesis indicated caveolae as an important mechanism of *S. aureus* pKikume for bacterial uptake and survival within HEp-2 epithelial cells, a potential role of *hla* for facilitating *S. aureus* internalization into these cells was investigated in more detail. Expression kinetics of *hla* by *S. aureus* after co-cultivation with HEP-2 cells for 30, 60, and 90 min showed a steady increase of *hla* expression over time (Fig. 43).

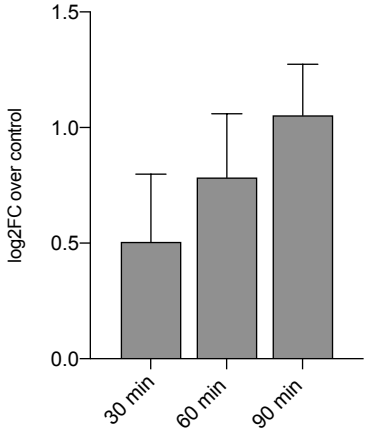

Figure 43 | Gene expression kinetic of hla in *S. aureus* pKikume after co-cultivation with HEp-2 cells using qRT-PCR.
Analysis of hla expression in *S. aureus* pKikume after co-cultivation with HEp-2 for 30, 60 and 90 min using qRT-PCR. Displayed as fold changes [log_2] over control *S. aureus* inoculum, each bar represents the average of 6 biological replicates ± SEM.

Hla was also detected at the protein level in the supernatant of HEp-2 cells co-cultured with *S. aureus* pKikume for 30, 60, 90, and 120 min by western blot with increasing concentration over time (Fig. 44). The band with a size of around 35-40 kDa depicted in the figure indicates the monomer of the hla protein.

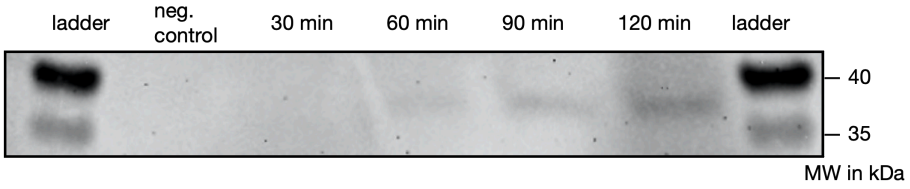

Figure 44 | Western blot analysis of hla in the HEp-2 – *S. aureus* co-culture supernatants.
Supernatants of HEp-2 cells co-cultured with *S. aureus* pKikume for 30, 60, 90 and 120 min were analyzed for secreted hla protein by western blot.

In order to investigate whether staphylococcal hla is involved in the internalization and/or proliferation process of *S. aureus* within HEp-2 cells, the capacity of a *S. aureus* strain deficient in the expression of hla (*S. aureus* SH1000 Δhla) to internalize and proliferate within HEp-2 cells was compared with that of *S. aureus* SH1000 wild type strain (WT). The burden of intracellular bacteria is lower in HEp-2 cells infected with *S. aureus* SH1000 Δhla than in HEp-2 cells infected with the WT strain at 2h and significantly at 4h p.I. (Fig. 45). Furthermore, a significant increase between 2h and 4h p.I. could be only obtained in epithelial cells infected with the WT strain (Fig. 45). These findings clearly demonstrate that the ability of *S. aureus* to invade, persist and multiply in epithelial cells is diminished by lacking the production of hla.

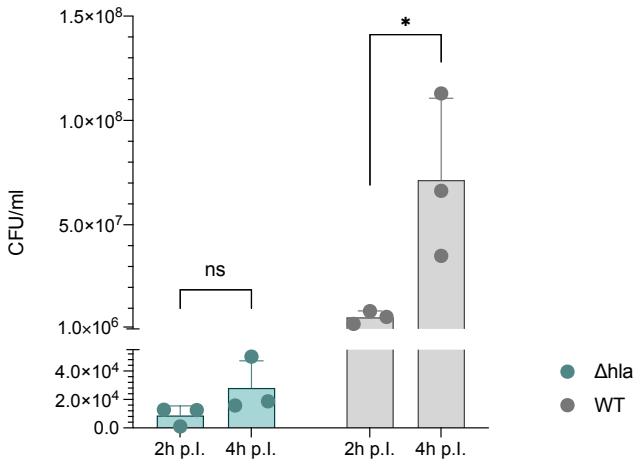

Figure 45 | Intracellular bacterial burden in HEp-2 cells infected with either *S. aureus* SH1000 WT or isogenic Δhla mutant strain.
Intracellular bacterial numbers in HEp-2 cells infected with either *S. aureus* SH1000 WT (grey bars) or Δhla (blue bars) were determined at 2h and 4h p.I. by plating serial dilution son blood agar plates. Each bar represents the average ± SD of biological triplicates; each symbol represents one biological replicate (n=3, Sidak's multiple comparisons test, * *p*-value < 0.05).

the influence of hla in the internalization of *S. aureus* within epithelial cells, HEp-2 cells either infected with the *S. aureus* SH100 WT strain or with *S. aureus* Δhla were examined by FESEM technology. (Fig. 46). The reduced capacity of *S. aureus* Δhla to internalize into the HEp-2 cells demonstrated by the platting experiments described above could be also confirmed (Fig. 46 b). After 2h infection with *S. aureus* Δhla, numerous bacteria are found adhered to the cell surface, forming bacterial aggregates and building grape-like structures. Only very few internalized bacteria and no cavity-like structures could be observed on the cell surface, indicating a lack of caveolae formation.

Together, these results support a role for Hla in the capacity of *S. aureus* to internalize within epithelial cells via caveosome formation.

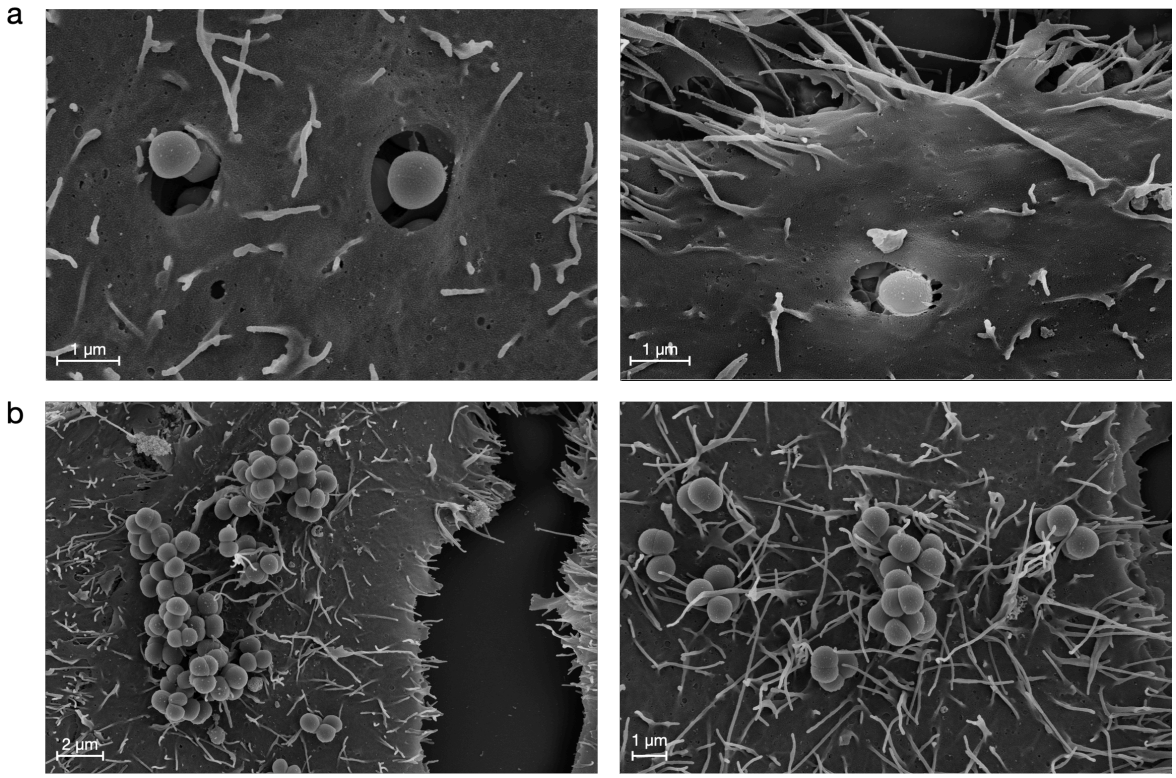

Figure 46 | Field-emission scanning electron microscopy (FESEM) of HEp-2 cells infected with *S. aureus* SH1000 WT or isogenic Δhla mutant strain.
Representative FESEM images of epithelial HEp-2 cells infected with *S. aureus* SH1000 WT (a), showing cavity-like structures for bacterial entry, or Δhla mutant strain (b), showing impaired bacterial uptake, for 2h.

To further corroborate the requirement of hla for *S. aureus* infection of epithelial cells, the supernatant of the hla-producing WT strain was collected after growth in cell culture medium for 2h followed by sterile filtration and used to replace those of the Δhla strain during infection. Thus, secreted factors of the WT strain, including Hla, were supplemented to the infection of epithelial cells with the *S. aureus* hla knockout strain. After infection of HEp-2 cells with *S. aureus* WT or *S. aureus* Δhla with or without substitution of their supernatant by WT supernatant, intracellular viable bacteria were determined by platting serial dilutions on blood agar plates. A schematic model of this experiment is shown in Fig. 47 a. As depicted in Fig. 47 b for one representative experiment, reconstitution with WT supernatant reverted the deficiency of *S. aureus* Δhla resulting in increased bacterial internalization and replication between 2h and 4h p.I. to a similar extent as the WT strain.

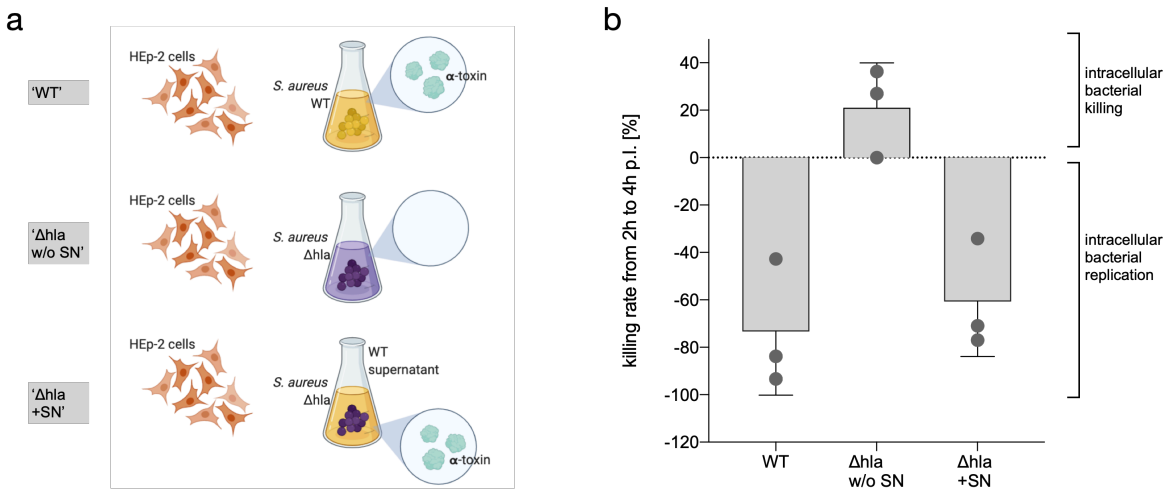

Figure 47 | Killing rates of *S. aureus* SH1000 WT, Δhla and Δhla substituted with WT supernatant in HEp-2 cells between 2h and 4h p.I.
(a) Schematic model of the substitution experiment (created with BioRender.com). (b) Intracellular killing activity [%] of HEp-2 cells between 2h and 4h p.I. with *S. aureus* SH1000 WT, its isogenic hla knockout mutant (Δhla) and the isogenic Δhla mutant substituted with WT supernatant substitution. Each bar represents the average ± SD of three technical replicates within one representative experiment out of three independent experiments.

These results demonstrate that the mechanism used by *S. aureus* to internalize within both, the human HEp-2 cells and murine HC-11 cells, and access an intracellular compartment permissive for intracellular replication is mediated by caveolae formation and induced by secreted staphylococcal toxin hla. Thus, inhibition of caveosome formation by MβCD pre-treatment on the host cell side or the inability of *S. aureus* mutant strains to produce staphylococcal alpha-toxin (Δhla strain), resulted in reduced bacterial burdens and impaired intracellular bacterial replication within epithelial cells.

5 DISCUSSION

S. aureus is a leading cause of bacterial infections since its initial discovery in 1881[8], in particular as nosocomial human pathogen[32,33]. Following a drastic reduction of infection numbers in the 20th century by the introduction of antibiotics as well as improvement of hygiene and social standards[403], we are now facing times where antibiotic treatment often fails due to the rapid and dramatic increase in antibiotic resistance development by pathogenic bacteria[404]. Antibiotic-resistant strains of *S. aureus* and especially methicillin-resistant *S. aureus* strains (MRSA), which are resistant to a number of β-lactam antibiotics, are frequent cause of infections in healthcare as well as community settings[404–408]. In 2017, the world health organization (WHO) published a list of bacteria for which new antibiotics are urgently needed and classified *S. aureus* as "high priority pathogen"[409]. Moreover, *S. aureus* is among the group of "ESKAPE" bacteria (acronym for *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species), which are the bacterial pathogens considered as the biggest concern for humanity by the Infectious Disease Society of America [410–412]. The therapeutic challenge posed to the clinicians by antibiotic-resistant *S. aureus* strains has intensified research on novel treatment modalities such as anti-virulence strategies, which are based on the attenuation of bacterial pathogens by the inhibition of virulence factors required for the establishment of infection[413]. The anti-virulence strategies are non-bactericidal and therefore exert low selective pressure on the microorganism resulting in reduced development of resistances[414]. Some of these strategies have already been designed targeting *S. aureus* adhesins[415], toxins[416], proteases[417,418] or regulatory systems[419–421] and have been tested in some animal models with promising results[422,423]. In addition, a big advantage of anti-virulence strategies compared to antibiotics is the specific targeting of the invading bacterial pathogen making them harmless for the commensal flora[424,425]. The development of these strategies requires a detailed knowledge of the virulence factors and survival pathways used by *S. aureus* to infect the host. The capacity of *S. aureus* to resist the killing mechanisms of phagocytic cells and survive intracellularly is an important feature of its pathogenesis[426–428].

Despite being classified as extracellular pathogen for decades, there is a growing body of evidence indicating that *S. aureus* can also invade and survive within a variety of non-professional and professional phagocytic cells[429–433]. Intracellular survival has been suggested a bacterial strategy to subvert immunological defense mechanisms, as well as extracellular bactericidal effects of antibiotics and may serve as a reservoir for chronic or relapsing staphylococcal infections[290,434,435]. Therefore, strategies to interfere with the capability of

S. aureus to survive intracellularly may be used as adjuvant therapy to treat staphylococcal infections. The mechanisms used by *S. aureus* to get into host cells have been shown to vary depending on the cell type and infecting *S. aureus* strain and can be either initiated by the host cell itself (e.g. phagocytosis, endocytosis) but can also be triggered by the pathogen[296,436]. Furthermore, it has been reported that there is heterogeneity within a population of host cells of the same cell type which may affect the permissiveness of intracellular survival of invading pathogens[437–440]. In addition, heterogeneity within the bacterial population often provides selective advantages to specific pathogen subpopulation during infection and the linked environmental changes[441]. These variations can either exist on a genetic level displayed by allelic variations or on a phenotypic level displayed by cell-to-cell differences in gene expression that enable the specific bacterial subpopulation to respond and adjust faster to the new environment. In this regard, it has been shown that in response to changes in environmental conditions, *S. aureus* rapidly modulate its gene expression pattern to adapt to the new host environment[442,443]. Depending on the site of infections and the specific host defense mechanisms that *S. aureus* is confronted with, the gene expression can differ drastically[444]. The bacterial adaption on the transcriptional level is mostly driven by stressors of the host immune response and the immune cells that are involved[445,446] and is critical for the bacterial survival[441,447]. The objective of this thesis was to investigate the transcriptional program activated by *S. aureus* to survive within macrophages and the potential influence of heterogeneity within these host cells to enable bacterial intracellular survival. Moreover, the influence of the internalization route on the capacity of *S. aureus* to survive within different host cells has also been investigated in the frame of this thesis.

A dual RNA-seq approach was used to investigate the simultaneous transcriptional responses of the internalized pathogen and the host cell in *S. aureus*-infected macrophages in combination with a *S. aureus* strain carrying a reporter system (*S. aureus* pKikume) that enable to differentiate between host cells harboring metabolically active/proliferating bacteria from those harboring bacteria with low metabolic activity.[326] *S. aureus* pKikume carries a photoconvertible fluorescence protein mKikumeGR that emits green fluorescence in its initial state and can be switched to emit red fluorescence by using a UV light pulse[327]. This irreversible system allows to correlate the recovery of green fluorescence with the metabolic activity and proliferation of the bacteria since recovering of green fluorescence is only possible through *de novo* production of fluorescent mKikumeGR protein combined with the dilution effect of the switched red fluorescence proteins in dividing cells. Flow cytometry analysis of *S. aureus* pKikume infected macrophages showed two populations at 4h p.I. with one macrophage population mainly harboring green fluorescent, metabolically active bacteria and the other

population mainly harboring red, metabolically inactive bacteria. These distinct host cell populations and their intracellular bacteria with different metabolic states were sorted according to the emitted fluorescence and analyzed by dual RNA-seq to determine bacterial and host factors underlying the different phenotypes.

No major differences were observed in the host transcriptional response between macrophages harboring mainly green and macrophages harboring mainly red bacteria, suggesting that the different metabolic states of internalized bacteria were not related to heterogeneity within the macrophage population. Both infected macrophages populations, independent of the metabolic status of the internalized bacteria, upregulated a set of inflammatory response-related genes similar to those already reported in the general response of innate immune cells to *S. aureus* infection[199,448,449]. Functional enrichment analysis of DEGs with increased expression in *S. aureus*-infected macrophages with respect to uninfected cells revealed enrichment of pathways associated with the immune system, infectious diseases and signaling pathways for environmental information processing. Enrichment in the 'cytokine-cytokine receptor interaction' and 'Toll-like receptor signaling pathway' in both macrophage subpopulations pointed towards the general recognition of *S. aureus* by macrophages via PRRs such as TLR2. These PRP's are able to recognize *S. aureus* cell wall components like LTA and peptidoglycan[200,201,349], activating an inflammatory response, including the expression of genes encoding pro-inflammatory cytokines (e.g. IL-6, IL-1β or TNF-α) and chemokines (e.g. Cxcl2, Ccl3 or Ccl9). Numerous interferon-related genes were also upregulated in macrophages in response to *S. aureus* infection that are most probably induced by an endosomal TLR2 signaling pathway as described by Stack *et al*[202]. Thus, after primary bacterial ligand binding on the cell surface, TLR2 receptors can be internalized into endosomal compartments where they induce the expression of IFN-β[203]. Moreover, TLR3 was also found upregulated in *S. aureus*-infected macrophages. Although this PRR has been functionally identified as a sensor of virus-derived dsRNA and its synthetic analog, polyriboinosinic:polyribocytidylic acid [poly(I:C)][450,451], some studies have reported that also structures other than dsRNA including virus-derived ssRNA with mismatched stems, RNA from necrotic cells, host cell mRNA and commensal bacterial dsRNA can activate TLR3[350,452–455]. Thus, it is possible that yet-unknown ligands from *S. aureus* will also activate TLR3.

Interestingly, enrichment of pathways associated with infectious disease were also observed and largely included pathways related to infections with intracellular pathogens such as 'Salmonella infection' or viruses such as 'HIV infection'. Therefore, it seems that *S. aureus*, which is considered an extracellular pathogen, can induce signaling pathways in macrophages that have been described for classically intracellular pathogens, emphasizing the ability of this bacterium to behave within host cells in a similar way than intracellular pathogens [456]. As

mentioned above, the different metabolic states of intracellular bacteria within macrophage subpopulations cannot be explained through the postulate that some host cells had better capacity to control phagocytized bacteria since no differences in gene expression were found between macrophages harboring mainly metabolically active and those harboring mainly metabolically inactive bacteria. For a more detailed study about the heterogeneity within a host cell population single cell sequencing should be also considered. Such a single cell dual RNA-Seq (scDual-Seq) study has been performed recently for Salmonella-infected macrophages providing a comprehensive view of individual cellular states[457]. In addition, there is a rapidly growing research area in immunology, called immunometabolism, which has been recently established to investigate the metabolic state and shifts of host cells in response to pathogens, since specific states of disease are displayed by host cell metabolism[458–460]. A second area closely connected is pathometabolism, a term for the complex metabolic adaptions of host cells and their intracellular pathogens during infection[461]. In both fields, single cell dual-RNA sequencing can provide a deeper understanding of the infection process and may uncover new targets for novel therapeutic applications.

The analysis of the transcriptional profile of intracellular *S. aureus* during infection of macrophages indicated significant differences in gene expression between red and green bacterial subpopulations. Nevertheless, both subpopulations up-regulated the expression of a set of common genes in comparison to the bacteria used for the inoculum which may be required for bacterial survival in the intracellular environment, independently of the metabolic status. This common response included genes related to the biosynthesis of amino acids, sugar transport systems, molecular chaperons, virulence factors, regulatory systems and the central metabolism. This indicates that after internalization within host cells, *S. aureus* intracellular survival highly depends on its capacity to acquire specific nutrients from the host environment through the expression of specific transport systems or *de novo* synthesis. As intracellular pathogens depend on the acquisition of nutrients from their host[461,462], they have evolved a great variety of mechanisms to get these nutrients, including alteration of host metabolic pathways, increased transport of nutrients or exploiting host mechanisms for macromolecule degradation[292,463–466]. The up-regulation of genes belonging to one of the major heat shock response protein systems DnaK, including the genes *grpE*, *dnaK*, *dnaJ* and their regulator *hrcA*, and GroESL[467–470] strengthen the assumption that *S. aureus* faces high levels of stress in the intracellular compartment where the bacteria is located since these heat shock proteins play an important role in stress tolerance. Different from what would be suggested by their names 'heat shock proteins' (Hsps), the expression of these genes can also be induced without thermal stress[471], shown by previous studies where a non-functional DnaK system resulted in

significantly impaired bacterial responses to oxidative stress or low pH[472–475]. Besides this group of classical Hsps that belong to class I chaperons, the genes coding for another group of Hsps classified as class III chaperons, including ClpC and ClpB[476], were found up-regulated in the transcriptional profile of intracellular *S. aureus*. CplC is a stress sensor of *S. aureus*, which play a role in stress resistance and together with ClpB is induced during acid shock[472,477–479]. Together all these chaperon genes indicate a stress response of intracellular *S. aureus* in both green and red subpopulations most probably induced by oxidative stress and low pH as it can be found in phagosomes of phagocytic cells like macrophages.

In addition, a set of genes encoding virulence factors such as the cytotoxins Hla, ɣ-hemolysin subunits and beta phenol-soluble modulins (PSM) were up-regulated by intracellular *S. aureus*. The role of Hla in the context of intercellular persistence and survival of *S. aureus* in various host cell types is rather controversial and highly depends of the host cell type. Thus, while in some studies it could be shown that Hla is involved in phagosomal escape and intracellular survival in macrophages[287] as well as a cystic fibrosis lung cell line[480], other studies have reported that Hla is not sufficient to lyse phagosomes in HeLa[481,482] and upper airway epithelial cells[483]. Also PSM*β* has been shown to play a role in phagosomal escape of intracellular *S. aureus* in gain of function assays[289] and PSM*α* in *in vivo* studies[137]. These incongruences may reflect a redundancy in the virulence factors used by *S. aureus* to survive and replicate within host cells in a strain- and host cell-dependent manner and underlines the complexity of this multifactorial process. Even the site of intracellular bacterial replication seems to be unclear, since some studies argues for, others against bacterial phagosomal escape and most *S. aureus* strains have been reported to be unable to escape into the cytosol of host cells[288]. The aforementioned virulence determinants are controlled by the most prominent regulatory system in *S. aureus,* the accessory gene regulator (Agr), that has been shown to be crucial for intracellular survival of *S. aureus* within macrophages[287]. The genes encoding the different components of Agr were upregulated by both green and red intracellular *S. aureus*. Other genes that were also upregulated by both intracellular bacterial populations encoding the metalloprotease aureolysin (*aur*) and the extracellular adherence protein (*eap/map*). Aureolysin may support the survival of *S. aureus* after phagocytosis since it is capable to inactivate the human defensin LL-37, and in general is required for full bacterial virulence[255]. In contrast, the role of Eap for the intracellular survival and replication of *S. aureus* remains undetermined. The same holds true for SSL11, the superantigen like protein 11, which has been shown to influence neutrophil functions including neutrophil activation[356,357] but so far no function for SSl11 in the intracellular milieu has been reported.

While the genes commonly upregulated by intracellular red or green *S. aureus* compared to inoculum bacteria represent the general response to the intracellular milieu, the

genes differentially expressed between intracellular green and red *S. aureus* subpopulations may represent the bacterial response to the environment within the specific compartment where they reside and may identify factors that facilitate better or worse intracellular survival. Thus, genes encoding determinants linked to protein synthesis, cell division, DNA replication and cell wall synthesis showed higher expression in intracellular green *S. aureus*, confirming the metabolic activity and proliferation of these bacterial. In contrast, the red metabolically inactive bacteria had higher expression of genes encoding factors involved in the oxidative stress response. These genes include catalase (*kat*), superoxide dismutase (*sod2*) and alkyl hydroperoxide reductase C (*ahpC*), most probably needed by the bacteria to neutralize reactive oxygen species present in the phagolysosomes in macrophages and survive within this acid environments[484] as well as genes coding for factors involved in degradation of misfolded proteins[284,485,486]. In this regard, it has been reported that after uptake of *S. aureus* by professional phagocytes initial phagosomal maturation is happening independent of the host species or the cell type[287,288,354,364,487]. However, in some cases phagolysosomes are not fully functional as key lysosomal components such as cathepsin D failed to be recruited to the *S. aureus* containing phagosome (SaCP)[282,487]. Another interesting gene differentially expressed between the green and the red intracellular *S. aureus* is the gene encoding the hibernation promoting factor SaHPF (*saHPF*) that was expressed to much greater extent by red fluorescent *S. aureus*. In general, ribosome silencing factors mediate the dimerization of 70S ribosomes into 100S complexes to inactivate them and thus suppress translation[362,488]. Since protein synthesis is one of the most energy-consuming processes[489–491], translational suppression is a common adaption mechanism to save energy in response to stress[362]. In comparison to the ribosome silencing factors found in other bacteria such as *E. coli*, the SaHPF is twice as long[492–495]. Furthermore, whereas 100S complexes of *E. coli* only formed during the stationary phase where nutrient availability is limited[496–498], in *S. aureus SaHPF*-mediated 100S complexes are observed in all growth phases, independently of nutrients availability[494,499]. Beside reducing energy consumption, the 100S complexes can be dissociated and recycled into active ribosomes even under nutrient starvation[362]. Little is known of the relevance of *SaHPF*-mediated 100S complexes during *S. aureus* infection, however, ribosome hibernation seems to be an important process for bacteria to enter a quiescence status and for subsequent resuscitation, and it has been reported that SaHPF is important for long-term *S. aureus* viability[362]. Furthermore, proteomics studies have shown that SaHPF is one of the predominant proteins expressed by *S. aureus* upon host cell internalization[500] as well as in an *in vivo* murine pneumonia model[501]. The observation that the gene encoding SaHPF is significantly higher expressed in intracellular red *S. aureus* suggests that, in contrast to the metabolically

active/replicating green bacteria, the red population may represent an intracellular quiescent long-term persistent bacterial reservoir. Thus, the potential role on the long-term survival of *S. aureus* within eukaryotic cells needs to be further investigated in future studies. Interestingly, expression of the genes encoding the alternative sigma factor gene *sigH* and the natural genetic competence genes *comEC* was higher in green *S. aureus*. SigH is a homologue to SigB of *B. subtilis* that was identified on a conserved gene cluster of the phylum *Firmicutes* that participates in a complex regulatory network of transcription controlling the expression of genetic competence genes including the *com* operon[168]. Natural genetic competence enables bacterial species to bind and internalize exogenous DNA[502], and incorporate it into their genome. SigH is not expressed under standard laboratory conditions and need particular nutritional requirements[168]. Since it is well known that genes encoding virulence determinants as well as genes encoding antibiotic resistances are spread among *S. aureus* via horizontal gene transfer[169], SigH-dependent development of natural competence in *S. aureus* could help to explain the acquisition of such factors[167]. Even though the frequency of DNA transfer is extremely low and transcriptional and post-transcriptional mechanisms limit SigH activity to only a small fraction of the bacterial population, the selective pressures would promote the survival and spread of individual bacteria that acquire anything beneficial[167]. Moreover, the uptake of exogenous DNA may also provide nutrients under starvation conditions[168,503], since some naturally transformable bacteria including *Haemophilus influenzae*[504], *Actinobacillus pleuropneumoniae*[505] and *Actinobacillus suis*[506] even rely on starvation to induce the expression of competence genes.

Because the pathway of internalization may influence the fate of intracellular *S. aureus,* the route used by *S. aureus* to internalize within macrophages was also investigated in the frame of this thesis using inhibitors to block specific routes and determining the consequences for *S. aureus* intracellular survival. Along with normal host cell phagocytosis, pathogen-induced internalization and caveosomal uptake of *S. aureus* into macrophages were investigated. Plating experiments confirmed the results of previous studies showing the capacity of *S. aureus* to replicate within professional phagocytes like macrophages and neutrophils[287,343,363,507]. Inhibition of the caveolae-mediated uptake did not affect the proliferation of internalized *S. aureus* indicating that caveolae are not the route used by *S. aureus* to gain access into a macrophage compartment which is permissive for bacterial replication. However, blocking of $\alpha 5 \beta 1$-integrin on the macrophage surface impaired intracellular bacterial replication between 2h and 4h p.I., emphasizing the importance of this pathway for *S. aureus* intracellular proliferation. Integrins are involved in various cellular processes that are linked to or required for remodeling of the actin cytoskeleton including integrin-dependent phagocytosis[508,509]. By targeting integrins on the host cell surface,

pathogens try to get in close contact with and often enter the host cell[510–512], especially via β1-integrins in a phagocytic zipper-like manner[509]. Well-known representatives that are hijacking the integrin receptor to promote their internalization are *Yersinia spp*[509,513,514]*. S. aureus* interacts with host $\alpha 5\beta 1$-integrin via fibronectin that form a bridge between the host cell $\alpha 5\beta 1$-integrin receptor on one side and the staphylococcal fibronectin binding proteins (FnBPs) on the other[367,515,516]. This mechanism has been found to be essential for *S. aureus* adhesion to or invasion of endothelial cells[517], epithelial cells[392,518] and fibroblastic cells[515] but it was not known if this mechanism was also used by *S. aureus* to internalize within phagocytic cells. Here, we could show that *S. aureus* utilizes $\alpha 5\beta 1$-integrins to internalize within macrophages and enter a subcellular compartment that allows bacterial proliferation and prevention of the classical endocytic pathway.

The importance of this pathway for the pathogenesis of *S. aureus* has been emphasized in previous studies showing the capacity of *S. aureus* to induced the expression of β1-integrins by the host cell through the secretion of Hla in order to enhance its own uptake [370,434]. FESEM examination of *S. aureus*-macrophage interaction showed extensive membrane ruffles at the site of bacteria interaction with the host cell surface but no evidence for caveolae formation confirming the results obtained with the specific inhibitors for $\alpha 5\beta 1$-integrin or cavaeolae-mediated pathways. Membrane ruffles are actin-rich membrane protrusions that play a role in cell migration[519], the uptake of external fluids[520] or phagocytic processes[521]. What special process or mechanism induced these membrane protrusions and whether or not this was a cellular driven classical phagocytosis or a pathogen-induced process like macropinocytosis cannot be clearly answered by the FESEM examination. Because several studies have shown that *S. aureus* survival within host cells is linked to its capacity to breach the phagocytic vacuole and escape into the host cytoplasm[145,289,427], the intracellular location of *S. aureus* within macrophages was investigated by TEM. *S. aureus*-infected macrophages pre-loaded with BSA-gold showed no evidence for phagosomal escape of *S. aureus* into the host *c*ytoplasm. These results are in agreement with several other studies such as those reported by Schröder *et al.*[286] or by Olivier *et al*.[522] In addition to TEM, examination of *S. aureus* by fluorescence microscopy indicated that most intracellular bacteria were located in compartments of the endocytic pathway fused with phagolysosomes. Nevertheless, some bacteria could be found in vacuole like compartments, which seems not to be linked to the endosomal pathway, similar to so called spacious vacuoles. Weather these structures can be defined for example also as non-functional parts of the endosomal or phagosome pathway remains to be elucidated in further studies.

Examination of the intracellular location of photoconverted green or red *S. aureus* by fluorescence microscopy indicated the presence of green replicating bacteria located in spacious vacuoles and the presence of red bacteria co-localized with endosomes in the same macrophage. A similar situation was described by Kubica *et al*. in *S. aureus*-infected human macrophages, with most bacteria located in tight vacuoles and others in large, spacious vacuoles[287]. These authors also reported that a *S. aureus* mutant strain deficient in the expression of Hla resided only in tight vacuoles and was quickly eradicated by macrophages[287]. These observations support the assumption that spacious vacuoles can be permissive for *S. aureus* replication within macrophages. An additional study reported the presence of these two types of vacuoles after phagocytosis of *S. aureus* in neutrophils and suggested that effective bacterial killing was only taking place within the tight phagosomes[343]. Moreover, the authors proposed that the observed spacious vacuoles resemble macropinosomes that form from membrane ruffles folding back to the cell surface[523]. Immune cells like macrophages are continuously probing their environment in a special process called constitutive macropinocytosis[524] which enables them to capture phagocytic targets to improve pathogen clearance[525]. Initiation of this induced form of macropinocytosis can be facilitated by increased concentrations of growth factors or microbe associated molecular patterns (MAMP)[526,527]. Another trigger for macropinocytosis is through cooperative signal transduction of $\alpha 5\beta 1$-integrins and receptor tyrosine kinases (RTKs), resulting in reorganization of the actin cytoskeleton and formation of circular dorsal ruffles (DR) on the plasma membrane[528]. Beside their function for macropinocytosis, DRs have been shown to play a role in trafficking of integrin or actin cytoskeleton remodeling during migration and invasion, however, their specific function is still unclear[528–530].

Together with the results reported by the mentioned studies, we hypothesize that *S. aureus* can internalize within macrophages following different routes. In this regard, the bacteria can be actively taken up by macrophages via the classical phagocytic pathway and localize within phagolysosomes where they are subjected to high levels of stress. In these compartments, the main activity of *S. aureus* is directed to the neutralization of antimicrobial mechanisms, reparation of damaged bacterial components and reduction of high energy demanding activities to enable its long-term intracellular persistence. These bacteria have low metabolic activity and after photoconversion remain red. On the other hand, *S. aureus* can avoid the phagocytic pathway of macrophages by inducing its own internalization within macrophages via $\alpha 5\beta 1$-integrins. In addition to the classical FnBPA-Fn-integrin axis, *S. aureus* might also facilitate its uptake into macrophages via Hla-induced integrin signaling resulting in dorsal ruffling and macropinocytosis[528]. This signaling pathway leads to the formation of dorsal ruffles resulting in macropinocytosis and the emergence of spacious vacuoles

containing *S. aureus*. These special subcellular compartments are less harsh than phagolysosomes and seem to provide an intracellular niche that is permissive for bacterial replication. *S. aureus* residing in these compartments are metabolically active and proliferating and, therefore, can fast recover the green fluorescence after photoconversion.

Macrophages display high phenotypic heterogeneity and plasticity. Classically activated M1 and alternatively activated M2 macrophages represent the two extremes of a continuum spectrum of macrophage subtypes with different polarization and diverse functions[209,211–214]. In contrast to *in vitro*-cultured macrophages, the composition of macrophage subtypes during *in vivo* infection is quite diverse and plastic since their polarization status changes in response to the specific cytokines and other inflammatory signals encountered at the site of infections. The influence of specific macrophage polarization subtypes on the fate of internalized bacteria has been already studied for classically intracellular pathogens such as *Chlamydia pneumoniae*[531], *Salmonella typhimurium*[532] or *Mycobacterium tuberculosis*[533,534]. Moreover, the polarization of macrophages can be actively modulated by intracellular pathogens such as *L. monocytogenes*[535] or *M. tuberculosis*[536] into a state that favors their intracellular survival or replication[537]. Because little is known about the influence of the specific macrophage polarization on the intracellular lifestyle and probably survival of *S. aureus*, this issue was investigated in this study. For this purpose, differentiated bone marrow-derived macrophages (BMDMs) were polarized *in vitro* into M1 and M2 phenotypes and used to investigate the intracellular fate of *S. aureus* pKikume. Similar to what has been observed with intracellular pathogens, M2-polarized macrophages exhibited a reduced antimicrobial capacity against *S. aureus*[538] and were more permissive for intracellular *S. aureus* replication than M1-polarized macrophages. Flow cytometry experiments indicated that intracellular *S. aureus* exhibited a higher metabolic activity within the M2-polarized macrophages than within M1-polarized macrophages. However, macrophages from a specific subtype can also be re-polarized into other subtypes (e.g. shifting back from M2 to M1 macrophages)[537,539]. To stabilize the M2-phenotype, the experimental setup used in this study includes a continuous exposure of macrophages to growth factors used for M2 polarization throughout the infection. However, gene expression analysis of infected M2 macrophages revealed upregulation of genes encoding inflammatory factors, such as IL-6, IL-1β or TNF-α, typical of M1 macrophage. These observations suggest that *S. aureus* infection induced a shift of M2-polarized subtype towards a M1-like phenotype, but without reaching a full M1 phenotype since the gene expression of inflammatory cytokines was, in generally, higher in infected pre-polarized M1 macrophages than in infected M2 macrophages.

The data obtained in this thesis indicate that, even under anti-inflammatory environmental conditions that favor macrophages polarization towards M2 phenotype, these cells shift to an M1-like inflammatory phenotype in response to *S. aureus* infection most probably to enhance the control of the pathogen. A similar situation has been reported by Buchacher *et al.*, which also observed increasing amounts of secreted pro-inflammatory cytokines in M2-polarized macrophages after infection with *C. pneumoniae*[531]. Nevertheless, the fact that intracellular *S. aureus* was able to survive and even replicate to a significantly higher extent within pre-polarized M2 macrophages than in pre-polarized M1 macrophages indicates that *S. aureus* encountered a less harsh environment in M2 macrophages. Taken together, these data giving strong evidence that in contrast to M1 macrophages, M2 macrophages display a hostile niche for intracellular replication of *S. aureus* and creating a window of opportunity for the pathogen. Since the homogeneity of *in vitro*-cultured macrophages does not reflect the heterogeneity of macrophages and their polarization during *in vivo* infection, further experiments using *in vivo* model systems in combination with tools like dual RNA-seq are necessary to fully understand the influence of macrophages polarization on the fate of intracellular *S. aureus*.

Before *S. aureus* comes into contact with macrophages, they first encounter epithelial cells lining the internal and external surfaces of the body, such as the skin or the respiratory tract[173,174]. With less efficacy than professional phagocytes, these non-professional phagocytic cells are also able to engulf and take up pathogens[181,182,540] and contribute to the initiation of the immune response to infection[178,181,541]. For this reason, the interactions of epithelial cells with *S. aureus* was also investigated in this study. Special emphasis was given to the intracellular bacterial survival and the uptake route that is permissive for bacterial proliferation. Using the same experimental approach that was applied for macrophages, human and mouse epithelial cells (HEp-2 and HC-11, respectively) were infected with *S. aureus* pKikume in absence or presence of special inhibitors for $\alpha 5\beta 1$-integrin-mediated or caveolae-mediated uptake. Determination of intracellular bacterial numbers revealed the capacity of *S. aureus* to replicate within these epithelial cells between 2h and 4h p.I. Interestingly, intracellular bacterial proliferation was impaired when caveolae formation was inhibited by either MβCD or Filipin III. In contrast to macrophages, the survival pathway of *S. aureus* within epithelial cells seems to be independent of the $\alpha 5\beta 1$-integrin receptor since blocking this receptor did not change the ability of *S. aureus* to replicate within HEp-2 cells. Hence, the route used by *S. aureus* to gain access into the intracellular milieu to survive and replicate is specific for the host cell-type.

Caveolae-mediated endocytosis has become of great interest since it could be demonstrated that this mechanism does not only take up macromolecules from the environment but also act as entry port for pathogens[374,542–547]. Furthermore, high quantities of membrane receptors, signaling molecules and membrane transporters can be found in these omega-shaped invaginations [375,548–550]. For host cells such as mast cells, epithelial cells or smooth muscle cells, caveolae represent signaling centers[551]. Internalization within eukaryotic cells using a clathrin-independent uptake via caveolae follows an endocytic pathway that enables pathogens to circumvent the classical endosomal/phagosomal-lysosomal route and, by this means, bypass the host cell degradation machinery[375]. In this regard, several studies have shown that pathogenic bacteria such as *Mycobacterium bovis*[552], *Chlamydia trachomatis*[546] or *Streptococcus pyogenes*[375] utilize caveolae to facilitate their entry into host cells. In this study, FESEM examinations of *S. aureus* infected HEp-2 cells show that *S. aureus* bacteria are mainly located in cavity-like invaginations, indicating the usage of caveolae by *S. aureus* to enter epithelial cells. This finding was confirmed by the impaired internalization of *S. aureus* within epithelial cells after disrupting caveolae formation using MβCD or Filipin III, resulting in bacterial aggregates attached to the host cell surface. In addition, TEM examination of *S. aureus* -infected HEp-2 cells that were pre-loaded with BSA-gold particles to label endosomal compartments showed mainly bacteria in membrane bound structures that were not associated with pre-labeled endosomes. These results suggest that *S. aureus* is located in a subcellular compartment within epithelial cells that does not end up in the classical endosomal-lysosomal pathway. To link the metabolic status of *S. aureus* pKikume with its capacity to survive and proliferate within epithelial cells, flow cytometry analysis identified a subset of cells harboring highly metabolic active staphylococci, increasing over the course of infection. However, to characterize this subset of *S. aureus*-infected cells in more detail, further experiments are needed to identify specific host or bacterial factors responsible for the high metabolic activity and replication of intracellular staphylococci.

In both cell types tested in this study, macrophages and epithelial cells, the uptake pathway used by *S. aureus* to enter a subcellular compartment where the bacteria are able to survive and replicate is unique and depending on the cell type the bacteria are encountering. However, no significant difference in the gene expression of staphylococcal virulence factors associated with either adhesion to host cells or involvement in the initial contact with these cells, including FnBPA, Map, ClfA and Hla could be obtained. This conclusion indicates no cell specific involvement in the adhesion and invasion process of *S. aureus* that can be linked to either the integrin-mediated uptake in macrophages or the caveolae-mediated uptake in epithelial cells on the gene expression level. Further studies focusing on protein level, for

example by using mutant strains might provide more information about the specific involvement of these factors in the cell-type specific adhesion and invasion process of *S. aureus*. Nevertheless, it is known from the literature that Hla elicits multiple cellular responses and has structural similarities to the cholera toxin of *Vibrio cholerae* which is known to bind to caveolae/lipid rafts[399–402]. Thus, the potential role of Hla for *S. aureus* internalization within HEp-2 cells via caveolae was investigated here using a *S. aureus* mutant strain (*S. aureus* Δhla) deficient in the expression of Hla. The numbers of intracellular *S. aureus* Δhla were significantly lower than WT bacteria and no intracellular replication of the Hla mutant within HEp-2 cells was observed. Furthermore, FESEM examination showed that the *S. aureus* Hla mutant was impaired to enter the epithelial cells, resulting in the agglomeration of numerous bacteria attached to the cells. In addition, no cavity-like invaginations were observed on the cell surface. The addition of supernatant of an Hla producing *S. aureus* strain (*S. aureus* SH1000 WT), containing secreted Hla, restored the intracellular proliferative capacity of the *S. aureus* hla mutant strain, corroborating thus the relevance of Hla for *S. aureus* survival and proliferation within epithelial cells. Together, these observations indicate a link between *S. aureus* internalization and replication within epithelial cells, the availability of caveolae/lipid rafts and the expression of Hla.

The mechanisms underlying these interactions have not been addressed in the frame of this study and deserve consideration in future research. In literature, the role of caveolae and clathrin-independent endocytic pathways as entry ports into the intracellular milieu for persisting pathogens has been discussed in recent years [553–558] and new technical opportunities can improve the ability to study such complex endocytic mechanisms in the future. Most likely, there are two different types of clathrin-independent endocytic cellular processes: macropinocytosis, already discussed above in context of a proliferation permissive uptake route in macrophages, and caveolar endocytosis[559]. Both pathways are suggested to involve lipid rafts[559–562], which are chemically unique domains in the plasma membrane containing high amounts of sphingolipids and cholesterols[563–565]. These areas within the plasma membrane are significant sites for signal transduction and endocytosis[566,567]. Interestingly, the staphylococcal Hla can bind to or interfere with specific receptors, including metalloproteinase ADAM10[104,568], $\alpha 5\beta 1$-integrins[569,570], anion exchanger 1 (AE1)[571], but also non-specifically to headgroups of special phospholipids of the plasma membrane, e.g. sphingomyelin[572–574]. These binding partners of Hla often can be found in such lipid rafts. In addition, the plasma membrane protein caveolin-1, frequently present in caveolae[562,575,576], has been found to also bind Hla[577]. Since caveolin-1 has no domain spanning into the extracellular space[577], it has been proposed that caveolin-1 stabilize the toxin within the membrane of the host cell after binding[107]. Beside the complexity about caveolae and endocytic trafficking within host cells, the multiple

functions of Hla including different modes of action of the monomeric or the heptameric form of the toxin as well as concentration dependencies are still discussed[112].

Taken together, the results of this study indicate that internalization of *S. aureus* into epithelial cells, bacterial intracellular survival and intracellular replication are dependent on the formation of caveolae/lipid rafts on the host cell surface as well as the secretion of Hla by the pathogen. Even though the internalization route permissive for *S. aureus* intracellular proliferation within macrophages mediated by $\alpha 5\beta 1$-integrin is different to the caveolae-mediated internalization route used by *S. aureus* in epithelial cells, both internalization pathways are clathrin-independent and incorporate signaling hubs within the plasma membrane of host cells to finally reach a subcellular compartment permissive for bacterial survival and proliferation.

A graphical summary of the proposed intracellular lifestyle of *S. aureus* within professional phagocytic macrophages (Fig. 48) and non-professional phagocytic epithelial cells (Fig. 49) with special focus on the specific internalization pathways used by *S. aureus* to enter host cells leading to permissive intracellular replication is shown below. As depicted in the left panel of Fig. 48, *S. aureus* can facilitate its own uptake into macrophages probably via a Hla-induced $\alpha 5\beta 1$-integrin signaling resulting in enhanced dorsal ruffling of the plasma membrane followed by macropinocytosis. The permissive environment within this spacious vacuole or macropinosome enable the metabolic active bacteria to survive and even replicate. However, macrophages can also internalize *S. aureus* into phagolysosomes via classical phagocytic pathways (Fig. 48, right panel). To survive within this harsh environment, *S. aureus* reduces its metabolic activity and probably enter a hibernation state that enable bacterial persistence. The uptake of *S. aureus* into epithelial cells, depicted in Fig. 49, that is permissive for intracellular replication is facilitated by the interaction of secreted Hla with caveolae/lipid rafts on the cell surface resulting in internalization via caveolar vesicles. Interestingly, both mechanisms are clathrin-independent suggesting that *S. aureus* is able to use this membrane enclosed compartments different from other clathrin-coated forms as a permissive niche for intracellular survival and replication.

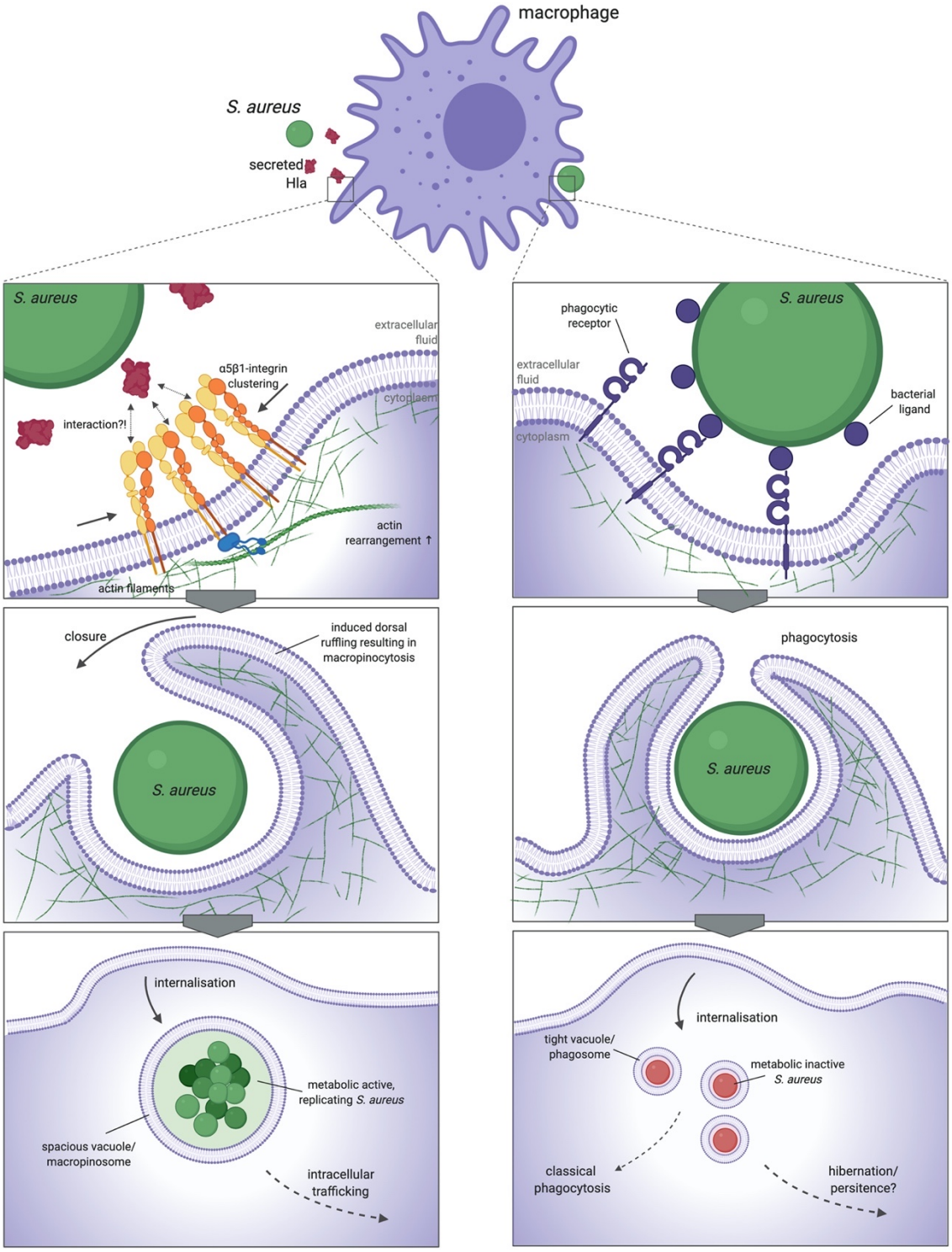

Figure 48 | Graphic summary of the proposed *S. aureus* uptake pathway in professional phagocytes (macrophages) that are permissive for intracellular survival and replication.
Created with BioRender.com

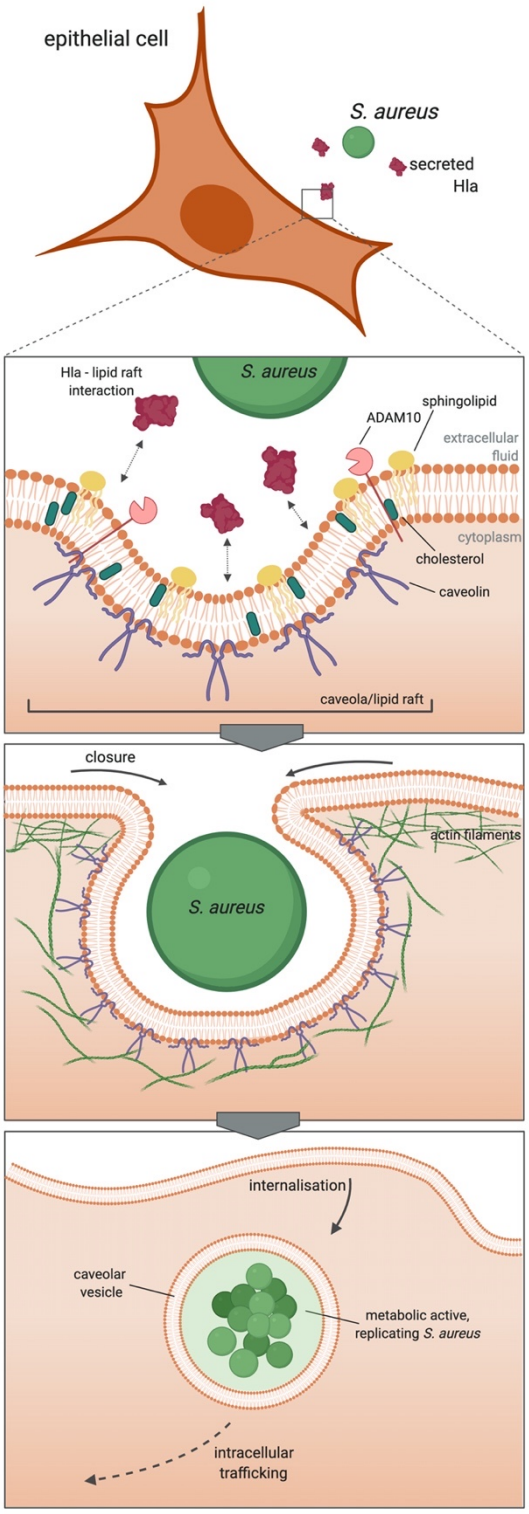

Figure 49 | Graphic summary of the proposed *S. aureus* uptake pathway in non-professional phagocytes (epithelial cells) that are permissive for intracellular survival and replication.
Created with BioRender.com

6 OUTLOOK

The results of this thesis demonstrate the capacity of *S. aureus* to use different pathways to internalize within host cells into subcellular, membrane-enclosed compartments permissive for intracellular survival and replication. These internalization pathways are clathrin-independent and mediated by either $\alpha 5\beta 1$-integrin triggered macropinocytosis in macrophages or by caveolae in epithelial cells. The physiological reason for this cell type-specific pathway for bacterial internalization is still unclear and needs to be addressed in further studies. While the findings of this study provide better understanding of *S. aureus* pathogenesis and first insights into the intracellular lifestyle and underlying mechanisms, several issues remain open and needs further investigations:

(I) The results of this thesis show that *S. aureus* resides and proliferates within intracellular compartments in both macrophages and epithelial cells and no evidence for a localization in the cytosol could be obtained. A more detailed characterization of the *S. aureus* containing vacuoles in macrophages as well as epithelial cells needs to be performed. In particular, to identify differences between the subcellular compartments harboring metabolically active/replicating *S. aureus* and those harboring metabolically inactive bacteria. This could be addressed using specific cell tracker dyes, different staining methods for membrane components, and super-resolution microcopy techniques.

(II) The results obtained from the transcriptome analysis give insights into host and pathogen gene expression at 4h p.I. To further investigate persistence of *S. aureus* within macrophages and epithelial cells and link the metabolic status of the intracellular bacteria with long-time intracellular survival, the monitoring period of infected cells should be extended. The specific role of genes identified to be differentially expressed between metabolically active/replicating and metabolically inactive bacteria needs to be validated by generating specific mutant strains.

(III) The findings of the transcriptome analysis are based on the sequencing of the entire subpopulation of macrophages harboring mainly green or red *S. aureus*. However, heterogeneity within these macrophage subpopulations is likely to be underestimated since bulk sequencing reflects only the average expression of genes in the total population. Heterogeneity of host gene expression during infection can be addressed

using single-cell RNA sequencing (scRNA-seq) of sorted subpopulations. This approach can help to identify the host factors that enable successful control and eradication of intracellular *S. aureus*.

(IV) The results of this thesis and others suggest spacious vacuoles within macrophages as intracellular location that is permissive for *S. aureus* proliferation. However, it remains unclear, what role the secreted Hla has on the formation or induction of these subcellular compartments. This question could be addressed by infecting macrophages with the *S. aureus* Δhla mutant strain and screen for the formation of spacious vacuoles using various microscopy techniques.

(V) A role for Hla in *S. aureus* internalization and intracellular proliferation within epithelial cells via caveolae has been clearly demonstrated in this thesis. Nevertheless, further investigations need to be performed in order to characterize the mode of action of Hla on the induction of caveolae pathway and to identify the interaction partners on the host cell surface in more detail.

(VI) Finally, while in the course of this thesis the established dual RNA-seq protocol including RNA extraction and bioinformatic analysis of the generated data was only applied to macrophages infected with *S. aureus* pKikume, the method could also be applied to *S. aureus* pKikume -infected M1 or M2 polarized macrophages as well as on sorted *S. aureus* pKikume -infected epithelial cells. Thus, key factors on both sides, the host and the invading *S. aureus*, that mediate intracellular survival/proliferation can be identified and further investigated using knock-out mutant strains of interesting candidate genes.

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8 APPENDIX

Table A1 | Representative DEGs of intracellular *S. aureus* (red or green) versus inoculum bacteria.

	Gene symbol	**Description**	**Green vs inoculum** [Log2 Fold Change]	**Red vs inoculum** [Log2 Fold Change]
Amino acids biosynthesis	*lysC*	aspartate kinase	5,29	6,43
	asd	aspartate semialdehyde dehydrogenase	4,96	5,98
	dapA	dihydrodipicolinate synthase	4,58	5,74
	dapB	dihydrodipicolinate reductase	4,86	5,79
	dapD	2,3,4,5-tetrahydropyridine-2-carboxylate N-succinyltransferase	4,53	5,45
	ilvA2	threonine dehydratase	3,2	3,63
	ilvB	acetolactate synthase large subunit	3,23	3,88
	ilvC	ketol-acid reductoisomerase	3,69	4,21
	ilvD	dihydroxy-acid dehydratase	2,74	3,45
	leuB	3-isopropylmalate dehydrogenase	3,18	3,61
	leuC	3-isopropylmalate isomerase large subunit	2,93	3,54
	leuD	3-isopropylmalate dehydratase small subunit	3,41	3,8
Transport systems	*gntP*	gluconate permease	3,26	3,86
	scrA	PTS system sucrose-specific transporter subunit IIBC	3,24	3,40
	opp-3A	oligopeptide ABC transporter substrate-binding protein	3,18	3,61
	opp-3F	oligopeptide ABC transporter ATP-binding protein	3,13	3,51
	glcB	PTS system glucose-specific transporter subunit IIABC	2,88	3,11
	malF	maltose ABC transporter permease	2,74	2,97
	metP2	ABC transporter permease	2,36	2,81

	Gene symbol	Description	Green vs inoculum [Log2 Fold Change]	Red vs inoculum [Log2 Fold Change]
Molecular chaperones/ Stress response	*danK*	molecular chaperone DnaK	2,94	3,91
	dnaJ	molecular chaperone DnaJ	1,91	2,62
	groES	co-chaperonin GroES	2,78	3,71
	grpE	heat shock protein GrpE	3,27	3,96
	groEL	chaperonin GroEL	3,11	4,16
	hrcA	Heat-inducible transcription repressor	3,25	4,24
	clpB	ATP-dependent Clp protease ATP-binding subunit ClpB	5,22	6,31
	clpC	endopeptidase	2,51	3,68
	clpL	ATP-dependent Clp protease ATP-binding subunit ClpC	1,55	1,85
Virulence factors and regulatory systems	*hlgA*	gamma-hemolysin h-gamma-II subunit	4,07	5,30
	hlgB	leukocidin f subunit	4,86	6,11
	hlgC	leukocidin s subunit	4,26	5,41
	hla	alpha-hemolysin	4,01	4,66
	psmβ1	Phenol soluble modulin beta 1	2,27	2,08
	psmβ2	phenol soluble modulin beta 2	3,55	2,99
	eap/map	MHC class II analog protein	2,20	2,32
	aur	zinc metalloproteinase aureolysin	2,07	2,88
	agrA	accessory gene regulator protein A	2,04	1,91
	agrB	accessory gene regulator protein B	1,91	1,64
	agrC	accessory gene regulator protein C	1,97	2,00
	ssl11	superantigen-like protein	1,67	-2,11
Central meta-bolism	*gapB*	glyceraldehyde 3-phosphate dehydrogenase 2	3,84	4,04
	pckA	phosphoenolpyruvate carboxykinase	3,49	3,66
	gntK	gluconate kinase	3,12	2,99

Table A2 | Representative DEGs of intracellular green vs red *S. aureus*.

Locus tag	Gene symbol	Description	baseMean	Green vs Red [Log2 Fold Change]	padj
SAOUHSC_00101	*deoB*	phosphopentomutase	227,38	-0,92	3,02E-03
SAOUHSC_00155	*glcA*	PTS system glucose-specific protein	206,97	0,80	2,31E-02
SAOUHSC_00183	*uhpT*	sugar phosphate antiporter	265,27	1,43	7,31E-04
SAOUHSC_00232	*lrgA*	murein hydrolase regulator LrgA	263,75	3,03	4,42E-05
SAOUHSC_00337	*mdh*	hypothetical	49,42	-0,97	3,35E-02
SAOUHSC_00365	*ahpC*	hypothetical	819,61	-0,98	2,37E-02
SAOUHSC_00399	*ssl11*	superantigen-like protein	19,99	3,79	9,32E-06
SAOUHSC_00427	*sle1*	autolysin	271,35	1,20	9,80E-03
SAOUHSC_00454	*holB*	DNA polymerase III subunit delta'	48,08	2,11	9,32E-06
SAOUHSC_00456	*yabA*	DNA replication intiation control protein YabA	34,27	1,14	2,24E-02
SAOUHSC_00502	*ctsR*	hypothetical	431,75	-1,28	7,14E-03
SAOUHSC_00504	*mcsB*	ATP:guanido phosphotransferase	923,94	-1,24	7,22E-03
SAOUHSC_00505	*clpC*	endopeptidase	2995,31	-1,19	2,09E-02
SAOUHSC_00515	*sigH*	hypothetical	29,40	1,19	3,27E-02
SAOUHSC_00707	*fruB*	1-phosphofructokinase	30,95	1,27	3,52E-03
SAOUHSC_00767	*saHPF*	hibernation factor	376,48	-2,00	3,64E-05
SAOUHSC_01031	*cydA*	cytochrome d ubiquinol oxidase subunit I	96,33	1,94	2,65E-05
SAOUHSC_01032	*cydB*	cytochrome d ubiquinol oxidase subunit II	69,21	1,82	3,56E-04
SAOUHSC_01063	*ftsW*	hypothetical	88,85	1,28	3,46E-04
SAOUHSC_01144	*ftsL*	cell division protein	31,81	1,85	8,05E-03
SAOUHSC_01145	*pbp1*	penicillin-binding protein 1	329,08	0,92	2,37E-02
SAOUHSC_01146	*mraY*	phospho-N-acetylmuramoyl-pentapeptide- transferase	55,81	1,81	1,79E-04
SAOUHSC_01147	*murD*	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase	116,32	1,19	2,09E-02

Locus tag	Gene symbol	Description	baseMean	Green vs Red [Log2 Fold Change]	padj
SAOUHSC_01148	*divIB*	cell division protein	153,63	0,93	8,93E-03
SAOUHSC_01149	*ftsA*	cell division protein	422,97	0,53	1,99E-02
SAOUHSC_01253	*spoIIIE*	hypothetical	243,55	1,15	1,94E-05
SAOUHSC_01327	*katA*	catalase	446,59	-1,09	1,77E-02
SAOUHSC_01337	*tkt*	transketolase	1038,62	-0,78	2,83E-02
SAOUHSC_01347	*citB*	aconitate hydratase	560,33	-0,97	7,36E-04
SAOUHSC_01653	*sodA*	superoxide dismutase	269,34	-0,78	3,09E-02
SAOUHSC_01691	*comEC*	DNA internalization-related competence protein ComEC/Rec2	32,38	0,99	1,97E-02
SAOUHSC_01801	*citC*	isocitrate dehydrogenase	159,38	-1,12	1,46E-02
SAOUHSC_01802	*citZ*	hypothetical	85,20	-0,98	7,31E-04
SAOUHSC_01857	*ftsK*	hypothetical	219,24	1,12	8,87E-05
SAOUHSC_02316	*cshA*	DEAD-box ATP dependent DNA helicase	515,07	1,25	9,32E-06
SAOUHSC_02343	*atpG*	F0F1 ATP synthase subunit gamma	514,55	0,81	3,54E-03
SAOUHSC_02345	*atpA*	F0F1 ATP synthase subunit alpha	999,26	0,99	1,47E-03
SAOUHSC_02346	*atpH*	F0F1 ATP synthase subunit delta	325,82	1,23	3,56E-04
SAOUHSC_02347	*atpF*	F0F1 ATP synthase subunit B	366,64	0,98	3,40E-02
SAOUHSC_02350	*atpB*	F0F1 ATP synthase subunit A	446,62	1,11	1,84E-04
SAOUHSC_02517	*topB*	DNA topoisomerase III	123,48	0,69	2,91E-02
SAOUHSC_02520	*glcU*	sugar transporter	96,64	1,11	4,45E-03
SAOUHSC_T00014	*trnaD*	tRNA-Asp	12,94	2,10	1,78E-02
SAOUHSC_T00023	*trnaG*	tRNA-Gly	19,09	4,06	1,84E-04
SAOUHSC_T00027	*trnaH*	tRNA-His	13,76	2,72	1,25E-02
SAOUHSC_T00031	*trnaL*	tRNA-Leu	28,77	3,07	1,45E-03
SAOUHSC_T00051	*trnaS*	tRNA-Ser	22,35	2,68	8,34E-04

Supplementary tables S1 – S5 are included on the attached electronic disc.

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